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• ORIGINAL RESEARCH •

Dysfunction of peripheral blood dendritic cells from patients with chronic hepatitis B virus infection

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Abstract

AIM **To identify the property of dendritic cells (DCs) of peripheral blood monocytes (PBMC) in patients with chronic HBV infection.**

METHODS **Twenty patients with persistent HBV infection were included in this study,** 10 **healthy subjects being used as a control group. The peripheral blood mononuclear cells (PBMC) of T cell-depleted populations were incubated and induced into mature dendritic cells in the RPMI-**1640 **medium in the presence of cytokines GM-CSF, IL-**4**, FLt-**3**, TNF-α and** 100**mL·L^{-1} of fetal calf serum for a total of** 10-12 **days. The expressions of surface markers on DCs were evaluated using flow cytometric analysis. ELISA method was used to determine the cytokine levels of interleukin-**12 **(IL-**12**) and IL-**10 **in the supernatant produced by DCs. For detection of the stimulatory capacity of DCs to T cell proliferation, mytomycin C-treated DC were incubated with allogenic T cells.**

RESULTS **A typical morphology of mature DCs from healthy subjects and HBV-infected patients was induced in *in vitro* incubation, but the proliferation ability and cellular number of DCs from HBV-infected patients significantly decreased compared with healthy individuals. In particular, the expression levels of HLA-DR, CD**80 **(B**7-1**) and CD**86 **(B**7-2**) on DC surface from patients were also lower than that from healthy individuals** (0.46 ***vs*** 0.92 **for HLA-DR,** 0.44 ***vs*** 0.88 **for CD**80 **and** 0.44 ***vs*** 0.84 **for CD**86**, *P*<**0.05**). The stimulatory capacity and production of IL-**12 **of DCs from patients in allogenic mixed lymphocyte reaction (AMLR) significantly decreased, but the production level of nitric oxide (NO) by DCs simultaneously increased compared with healthy subjects** (86±15 ***vs*** 170±22 **μmol·L^{-1}, *P*<**0.05**).

CONCLUSION **The patients with chronic HBV infection have the defective function and immature phenotype of dendritic cells, which may be associated with the inability of efficient presentation of HBV antigens to host immune system for the clearance of HBV.**

Subject headings dendritic cells/pathology; hepatitis B, chronic/pathology; nitric oxide/analysis; interleukin-10/ analysis; interleuki-12/analysis

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INTRODUCTION

The infection of hepatitis B virus (HBV) causes 130 million HBV carriers, of them 23 million were patients with chronic hepatitis B in mainland China. As a result, a considerable number of the patients are developing progressive liver diseases such as liver cirrhosis and hepatocellular carcinoma (HCC) each year[1]. The pathogenesis of developing chronic HBV infection is complex and unclear[2]. The dominant reason responsible for viral persistence within host is mainly lack of efficient antiviral immune response to the viral antigens, in particular, inability to produce specific CTL response, which is critical for complete elimination of virus within hepatocytes[3,4]. The mechanism for weak antiviral immune reaction in patients is not well understood. Dendritic cells (DCs) are one of the most potent antigen-presenting cells (APC) and play crucial roles in the enhancement or regulation of antiviral immune reactions. The previous reports showed the defects of DCs function in HBV-transgenic mice and in patients with HCC and chronic HCV infections[5-13], but there is little evidence of defect DCs function in chronic HBV-infected patients. In order to have more insights in this regard, we induced peripheral blood monocytes (PBMC) from HBV-infected patients and uninfected healthy donors into maturation of DCs, compared their morphologic characterization, expression of surface antigens, and their proliferation capability to stimulate AMLR for evaluation of the functions of DCs.

MATERIALS AND METHODS

Patients

Blood was obtained from each of the twenty patients who were positive for HBV antigens and serum HBV-DNA in this study after written consent had been obtained from them. All patients were negative for HCV infection and had no histories of other types of liver diseases. The diagnosis of chronic HBV infection is made in accordance with the standards for chronic viral hepatitis issued in the Fifth National Conference on Infectious Diseases and Parasitosis in China (Beijing, China, 1995)[14]. Ten age-matched healthy donors from the Blood Center of Chinese PLA 307 Hospital were assigned as controls. The clinical background of patients and donors are shown in Table 1.

Table 1 Clinical background of HBV-infected patients and healthy donors

Group	*n*	Gender M/F	Age (years)	ALT (nkat·L^{-1})	AST (nkat·L^{-1})	HBV DNA	HBsAg	Anti-HBs	HBeAg	Anti-HBe	Anti-HBc
Patients	20	17/3	29.5(12-47)	215±218	186±172	19	18	—	16	4	20
Donors	10	8/2	27.5(20-35)	20-40	20-40	—	—	6	—	—	—

—: Nagative results

Reagents

RPMI1640 medium, recombinant human IL-4, GM-CSF, fetal calf serum and TNF-α were all purchased from Gibco Co. (Jingmei Biological Co, Beijing). The Flt-3 ligand, mouse anti-human FITC-conjugated HLA-DR-FITC, CD-86-FITC, PE-conjugated CD80-PE, CD1α-PE monoclonal antibodies were obtained from BD-Pharmingen (Jingmei Biological Co, Beijing).

Culture of DCs

Fifteen milliliters of peripheral blood was drawn from each study subject in heparin-coated tube, PBMC were obtained by Ficoll Hypaque separation, washed twice, resuspended in RPMI1640 medium and incubated in 6-well culture plates at the concentration of 3×10^9 cells·L^{-1} in medium at 37℃ in 50mL·L^{-1} CO_2 atmosphere for 2h. Non-adherent lymphocytes were collected by careful rinsing in 37℃ warm and cryopreserved for their future use in antigen presentation assays. The adherent aggregates were kept for incubation in complete medium with 100mL·L^{-1} fetal calf serum (FCS) in the presence of 1×10^6 IU·L^{-1} GM-CSF, 1×10^6 IU·L^{-1} IL-4, and 50μg·L^{-1} Flt3-L respectively, and expanded over the next 7. At d7, 1×10^6 IU·L^{-1} TNF-α cytokine was added into the medium for 10-12 days incubation. The wells were fed every other day by aspirating 0.3mL medium and adding back 0.5mL fresh medium with cytokines. The protocol has been proven reproducible in 20 HBV-infected patients and 10 healthy donors[15].

AMLR stimulated by DCs

To evaluate the allo-stimulatory activity of DC, AMLR (allogeneic-mixed lymphocyte reaction) was performed[16]. After DCs had been treated with 50μg mytomycin C for 45 min at 37℃ and washed three times with 37℃ warm PBS (pH 7.0). The DCs were placed at 0.5×10^4, 1.0×10^4, 1.5×10^4 and 5.0×10^4 per well on 96-well flat-bottom culture plates, incubated in complete medium. T-cells were from the PBMC of a healthy donor by removing the CD8-positive cells, monocytes, B cells and NK cells with magnetic bead-tagged mouse monoclonal anti-human CD8, CD14, CD19, and CD56 Abs (BD-PharMingen). After the separation, the degrees of positivity of these cells in the samples were all less than 5% and those of CD4-positive cells were more than 95%, respectively. CD4 T cells at 2×10^5/well were mixed with DC and cultured for 4d at 37℃, 50mL·L^{-1} CO_2 atmosphere. The MTT method was used according to the manufacturer instructions.

Detection of NO IL-10 and IL-12

The levels of nitric oxide (NO) and cytokine production by DCs were determined according to a previous report with slight modification (Kakumu *et al*, 2000). In short, DC (1×10^9·L^{-1}), enriched from HBV-infected patients and uninfected healthy donors by cytokines, were cultured in RPMI1640 plus 100mL·L^{-1} FCS for 48h in the presence of *Staphylococcus aureus Cowan* I strain (SAC). After the end of the culture, supernatant was collected and centrifuged. The production of NO and cytokines in the culture supernatants was determined[16,17]. The levels of NO production in the sample was determined by assaying the stable end product NO^{2-} by Greiss reaction using a commercial kit (Griess Assay Kit NO kit-C; Wako), as described by Lu[18]. Aliquots of culture supernatants were incubated with Griess reagent (10g·L^{-1} sulfanilamide, 1g·L^{-1} nephthylethlenediamine, dihydrochloride, and 25g·L^{-1} H_3PO_4) 100 μL at room temperature for 10min. The color developed due to enzymatic reaction was determined with an ELISA reader at 540nm. Concentrations of NO in the sample were caliberated with a reference standard of sodium nitrite supplied with the kit and the levels of NO were expressed as μmol·L^{-1}.

IL-10 and IL-12 in the supernatants were estimated by an ELISA method using commercial kit (PharMingen, San Diego, CA) according to the instructions of the manufacturer. Samples were incubated on microtitre plates coated with the respective monoclonal antibodies (mAbs), followed by addition of a biotinylated second antibody. After removal of excess antibody, color development was finished by enzymatic reaction of strepavidin peroxidase, the intensity of which was directly proportional to the concentration of the respective cytokines in the samples. The amounts of cytokines in the samples were estimated by caliberating the absorbance (optical density, OD) values of the samples with A values of the standards, supplied with kits using an ELISA reader (Labsystems Multiskan MS, USA). The lowest levels of cytokines detectable by these kits were IL-10>15.0ng·L^{-1}, and IL-12>7.8ng·L^{-1}.

Flow cytometry

After 12d incubation, the mature DCs from patients and healthy donors were washed in PBS and directly stained with an optimum dilution of FITC conjugated specific antibody (PharMingen, San Diego, CA) or stained with an optimum dilution of primary antibody followed by FITC-conjugated antibody against HLA-DR, CD86 and PE-labelled antibody against CD80 and CD1a. Finally, FITC+ and PE-cells were counted in an FACScaliber (Becton Dickinson)[19,20].Subclass matched FITC-conjugated mouse IgG and FITC-conjugated secondary antibody alone served as controls.

Statistical analysis

The Student's *t* test was used to determine significant differences in mean values between two groups. Statistical significance was established at the $P<0.05$ level.

RESULTS

Proliferation and Morphology of DCs

The adherent cells, the progenitor cells of DCs were incubated in complete medium containing 100mL·L^{-1} FCS, Flt-3-L, GM-CSF and IL-4 at 37℃ in 50mL·L^{-1} CO_2 atmosphere. The proliferation of DCs could be observed from the third day

incubation. The number of proliferative DCs increased following the extension of incubation time, and reached top value at the 12th day, then gradually went down as shown in Figure 1. Under phase-contrast microscopy, the DCs display a typical morphology with many fine dendrites. These cells extend large, delicate processes or veils in many directions from the body, and display typical morphology of DCs. The yield of proliferation of DC populations increased around 45 times for the healthy donors and 30 times for HBV-infected patients after 12d incubation.

Surface markers on DC

Mature DCs 1×10^6 after 12d incubation were defined by the expression of dendritic cell-restricted markers. The analyses of flow cytometry showed that positive cells constituted between 56% and 80% of all cells recovered at the end of the culture; the purity was not different between patients and controls. The frequencies of $CD86^+$ cells in the dendritic cell population were 84%±10% for control donors (n = 10) and 44% ± 8% for patients with chronic HBV infection (n = 20), respectively, indicating a significant difference between the groups. In addition, the average ratio of $HLA\text{-}DR^+$, and $CD80^+$ surface molecules expressed in the dendritic cell population are shown in detail in Table 2. Figure 2 shows the fluorescence intensity of representative DCs from one patient and one healthy donor, and there was a high expression of surface markers on DCs from healthy donors, reflecting a significant statistical difference from the patient group.

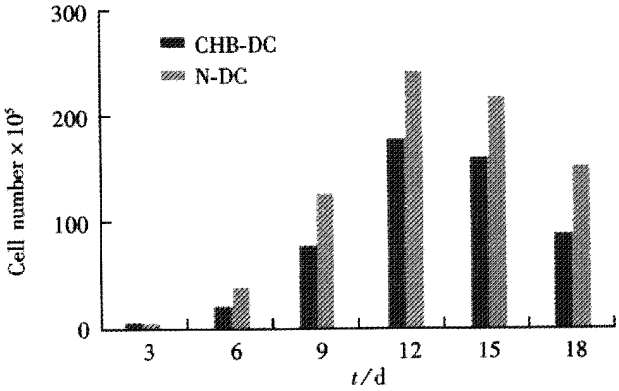

Figure 1 Proliferation of dendritic cell population in *in vitro* culture.

Table 2 Comparison of expression of DC surface markers from CHB patients and healthy donors (number fraction, $\bar{x}\pm s$)

Group	*n*	B7-1(CD80)	B7-2(CD86)	CD1[a]	HLA-DR
CHB-DC	20	0.44±0.08	0.44±0.08	0.25±0.07	0.46±0.09
Normal-DC	10	0.88±0.10[b]	0.84±0.09[b]	0.89±0.09[b]	0.92±0.09[b]

[b]P<0.001 *vs* CHB-DC.

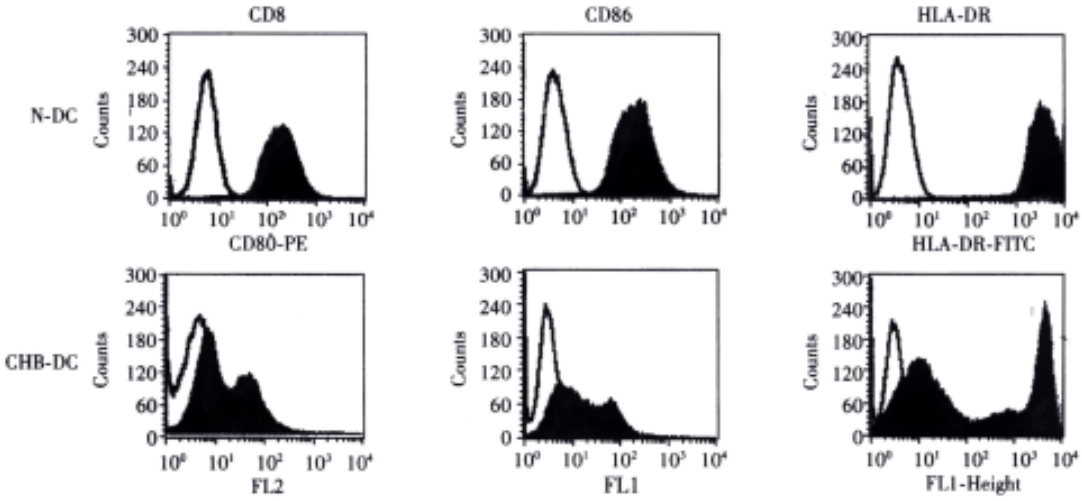

Figure 2 Comparison of fluorescent intensities of flow cytometric analyses on surface of dendritic cells (DCs) from a representative of HBV-infected patients (HB-DC) and a representative of uninfected healthy donors (N-DC). The green line is for signals from controls (DCs stained with mouse IgG_2 instead of specific antibody against surface marker of DCs). The green line with purple color represents the signals obtained from surface of DCs stained with corresponding specific antibody. Note the shift to the left in the peak with increasing expression of surface markers on DCs. Two other independent experiments gave similar results.

AMLR from HBV-infected patients

The T-cell stimulatory activity of DC populations in AMLR was expressed as stimulation index (SI) value, which is the ratio between the proliferative response (optical absorbance, OD) of T cells in the presence and the absence of DCs in the cultures at a T cell:DC ratio of 100:2.5, 100:5, and 100:25, respectively. Although the number of T cells was the same (2×10^5 cells/well) in all AMLR cultures, the SI went up significantly according to the increase of DCs number in AMLR. The SI values were between 2.1 and 4.8 for HBV-infected patients as compared with the values between 6.8 and 12.8 for healthy donors. The SI for HBV-infected patients was the lowest (SI=2.1) when the AMLR system contained 0.5×10^4 $well^{-1}$ of DCs. The results showed that HBV-infected patients tended to have significantly decreased T cell-stimulatory activity (P<0.01) as compared with the values for healthy donors.

Cytokine production by DC in AMLR

IL-12 is known to induce T cell proliferation and is the only

vital cytokine produced by DCs. Spontaneous IL-12 production of dendritic cells in the supernatants of dendritic cell population and AMLR are shown in Table 3. IL-12 produced by DC from patients (27±4ng·L^{-1}) was significantly lower than that from healthy donors (86±32ng·L^{-1}), indicating the statistical difference. Although the IL-12 in this AMLR was most likely produced entirely by the DCs, we decided to confirm this by culturing DCs for 48h and measuring the spontaneous production of IL-12 in culture by pure populations of DCs. Pure DCs from patients produced significantly lower amounts of IL-12 as against healthy donors. In addition, there was no statistical difference in IL-10 levels in AMLR supernatants of DCs from HBV-infected patients (18 ± 7 ng·L^{-1}) (Table 3) and normal controls (25±9ng·L^{-1}).

Table 3 Spontaneous cytokine production in pure DCs population and AMLR ($\bar{x}$±s, ng·L^{-1})

Dendritic cell	Supernatant from MLR culture		Supernatant from pure DCs population(ng·L^{-1})
	IL-12(ng·L^{-1})	IL-10(ng·L^{-1})	
CH-B	27±4	18±7	99±51
Normal	86±32[b]	25±9	218±104[b]

[b]P<0.01, *vs* Normal.

Increased NO production by DC from HBV-infected patients

As shown in Figure 3, the levels of NO in the AMLR supernatant of DCs from HBV-infected patients (168±35 μmol·L^{-1}) were significantly higher than that produced in AMLR of DCs from normal controls (90 ± 43 μmol·L^{-1}) (P<0.05). There was no correlation between the level of NO and the levels of transaminase (data not shown). NO produced by DCs is supposed to be one of the main products in AMLR supernatant. To have direct evidence of increased NO production by DCs, we cultured DC with SAC, a known inducer of NO. Pure DCs from HBV-infected patients produced significantly higher amounts of NO (170 ± 22 μmol·L^{-1}) than the normal controls (86 ± 15 μmol·L^{-1}, P<0.05, Figure 3). The significance of increased production of NO by DC in HBV infection might find the clinical implications of these observations.

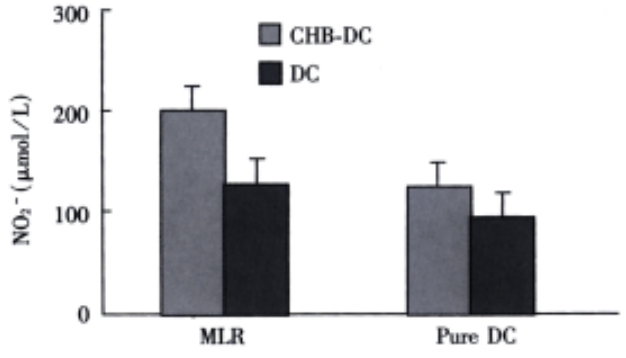

Figure 3 Increased NO production by DCs from HBV-infected patients. All the T cells (2×10^5/ well) were donated from a healthy volunteer in AMLR DC (1×10^4 well^{-1}) from patients with chronic hepatitis type B (CHB) and healthy donors were cultured for 5d. Pure population of DCs (1×10^9·L^{-1}) isolated by the cytokine enrichment method were cultured with SAC I strain 75mg·L^{-1} for 48h.

DISCUSSION

Though B and T lymphocytes are the main mediators of immunity, their function is under the control of DCs which is the sentinels of host immune system[21]. In this way, the property and its implications of DCs have become the focus of immunity against tumor and infectious diseases. Interestingly, the present culture techniques allowing the generation of large numbers of immunostimulatory DCs *in vitro* from human peripheral blood have made it possible to analyse the DC function in human diseases[7,10]. Though depletion and dysfunction of DCs were reported in some infectious diseases induced by HCV, HIV-1 and measles virus[6,20,22], dendritic cell function still remains uncertain in chronically HBV-infected patients. In particular, there appears to have few reports in the published literature about the effects of chronic HBV infection on DC function. These make us attempt to compare the stimulatory potential against allogenic CD4 T cells, levels of surface antigens, levels of interleukin-12, and degree of maturation of DCs from HBV-infected individuals with uninfected healthy donors in this study.

Flow cytometry showed that the expressions of cellular surface markers such as the co-stimulatory factors B7-1, B7-2 and CD1a and MHC II molecules HLA-DR on DCs significantly decreased in chronic HBV-infected patients compared to those in healthy donors. Furthermore, the T-cell stimulatory activities of DCs from patients were much lower than the DCs from uninfected healthy donors in AMLR.Simultaneously, there were notably low levels of cytokine IL-12 and high levels of NO produced by DCs from patients, in particular, NO was considered to have the suppressive activity on cellular enzymes and produce damage to normal cells. Taken together, our present study indicated that there is an immature phenotype and dysfunction of DC population in chronic HBV-infected patients in comparison with uninfected controls. It is necessary to study whether the dysfunction of DC population in patients is associated with the failure to mount an effective immune response for clearance of HBV.

Since IL-12 is an important cytokine to stimulate the proliferation of T lymphocytes, the decrease of IL-12 production was directly attributed to the low T-cell stimulatory of DCs from HBV-infected patients in AMLR[23]. The following reasons probably resulted in the low level of IL-12 production: ① IL-10 could inhibit the expression of class II and B7 molecules and suppress the IL-12 production of DCs[8]; ② there is a high level type I interferon in patients with HBV infection, which induces the down-regulation of IL-12 secretion of DCs[24]; ③ the previous study reported that the HBV itself and some cytokines induced by HBV infection may produce direct or indirect influences on transcriptions of IL-12 gene[25,26]; and ④ host genetic factors, such as the polymorphisms of both antigen-capturing receptor alleles and cytokine genes in DCs might explain HBV-infected patients mount dysfunction of DCs[27].

The maturation and efficient antigen-presentation of DCs are crucial for the initiation of immunity against viral infection[4]. The impairment of dendritic cell function suggested a role in the pathogenesis of chronic HBV infection, which has been probably attributed to the inability of the host to eradicate viruses[1]. It is unknown whether the HBV-infected DCs were eliminated by specific cytotoxic T lymphocytes (CTLs) if the DC is infected itself, which induced the dysfunction of DCs in these patients with chronic HBV infection or the HBV-infected DCs directly induce the T cell tolerance to HBV infection[27].

Decreased function of DC may allow the development of HBV infection, so that modulating the function of dendritic cells is considered to be beneficial to the production of

efficient immunity against viruses[28], but it must be made clear whether the dysfunction of DCs in patients is the result of direct injury of DCs from chronic HBV infection, or a protection from host itself to avoid the destruction of a large amount of HBV-infected hepatocytes induced by itself-inducing immune reaction, and or both In the early stage of HBV-infected patients, efficient antigen-presentation of DCs help the host to clear the virus[29], but in the late stage of chronic HBV-infected patients, induction of dysfunction of DCs or immune tolerance to HBV infection might also be helpful to host survival. Further studies are needed to clarify the mechanisms of depression of DCs function in HBV-infected patients[30,31].

The development of methods to generate a large number of DCs has facilitated their application for immunotherapy. Recent studies have demonstrated the safety and immunogenicity of DCs in humans and have begun to outline the durability, kinetics, and nature of the elicited T-cell responses. However, DC-based immunotherapy remains a challenge and several parameters need to be examined to optimize immune responses, in order to maximize the clinical efficacy against cancer and infectious diseases.

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