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Study on the distribution of CD8+ memory T cell subsets and IFN-γ level during the spontaneous clearance of hepatitis B virus in patients with chronic hepatitis B virus infection

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Abstract. – OBJECTIVE: To study the alteration of CD8+ memory T cell subsets under different immune statuses during the spontaneous clearance of hepatitis B virus (HBV) in Chinese patients with chronic HBV infection.

PATIENTS AND METHODS: We analyzed Chinese patients with chronic HBV infection including 10 patients with Hepatitis B surface Antigen (HBsAg) spontaneous seroconversion, 25 patients with Hepatitis B virus e Antigen (HBeAg) spontaneous seroconversion, 25 patients with chronic hepatitis B (CHB), and 25 chronic HBV carriers. The CD8+ T cells in peripheral blood were isolated, and flow cytometry was used to determine the percent change of CD8+ T memory cell subsets. ELISA was used to measure the levels of Interferon-γ (IFN-γ) secretion from CD8+ T cells.

RESULTS: (1) The percentage of CD8+ TN cells in peripheral blood was lower in the HBsAg seroconversion group than in the HBeAg seroconversion group ($p<0.01$), and higher in the CHB group and chronic HBV carrier group ($p<0.01$, 0.01); (2) The percentage of CD8+ TEM-2 memory T cells in peripheral blood was higher in the HBsAg seroconversion group than the HBeAg seroconversion group ($p<0.05$), CHB group, and chronic HBV carrier group ($p<0.01$, 0.01); (3) The percentage of CD8+ TEM-1 and CD8+ TCM cells in peripheral blood was higher in the CHB group and HBV carrier group than the HBsAg seroconversion group and HBeAg group, but there were no significant differences between groups ($p>0.05$); (4) IFN-γ production from CD8+ T cells in peripheral blood was higher in the HBsAg seroconversion group than the HBeAg seroconversion group ($p<0.05$), CHB group, and chronic HBV carrier group ($p<0.05$, 0.01).

CONCLUSIONS: The consistent increase of CD8+ TEM-2 cell subsets may be an important cause of spontaneous clearance of HBV. The disorder of CD8+ memory T cell differentiation may be an important mechanism of chronic HBV infection.

Key Words:
Memory T cells, CD8+, HBeAg, Hepatitis B, Chronic, CD45RA, CCR7.

Introduction

Studies have shown that CD8+ memory T cells (Tm) may be involved in the process of chronic hepatitis B virus (HBV) infection[1,2]. According to the expression of the chemokine receptors, CD45RA and CCR7, CD8+ memory T cells are divided into naive T cells (TN, $CD45RA^+CCR7^+$), central memory T cells (TCM, $CD45RA^-CCR7^+$), effector memory T cells (TEM-1, $CD45RA^-CCR7^-$), terminal effector memory T cells (TEM-2, $CD45RA^+CCR7^-$)[3,4]. In the present study, we observed the differentiation of CD8+ memory T cell subsets in the peripheral blood of patients with chronic HBV infection after spontaneous clearance of HBV, to explore the possible mechanism of HBV clearance in chronic HBV infection[5].

Patients and Methods

Patients

We analyzed patients with chronic HBV infection undergoing outpatient or inpatient treatment at Guangdong General Hospital from January 2013 to January 2015. None of the patients

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were treated with interferon, nucleoside analogs, or immunomodulators. A total of 35 patients with spontaneous seroconversion from HBV DNA-positive to negative during treatment were divided into groups. There were 25 patients with HBeAg spontaneous seroconversion (HBeAg seroconversion group) and 10 patients with HBsAg spontaneous seroconversion (HBsAg seroconversion group). Twenty-five chronic hepatitis B patients (CHB group) and 25 chronic HBV carriers (HBV carrier group) were selected as the control groups. The study received the approval from the Medical Ethical Committee of the Guangdong General Hospital.

Diagnostic Criteria

HBeAg spontaneous seroconversion: HBsAg was detected as positive, HBeAg was positive, and HBV DNA was positive 1 year before the study started. After 1 year, HBsAg was detected as positive, HBeAb was positive, and HBeAg and HBV DNA were both negative. HBsAg spontaneous seroconversion: HBsAg was detected as positive or negative, and HBV DNA was positive 1 year before the study started. After 1 year, HBsAb was detected as positive, and HBsAg, HBeAg, and HBV DNA were all negative. The diagnostic criteria for chronic hepatitis B infection and chronic HBV carrier were according to the "The Guidelines of the Prevention and Control of Chronic Hepatitis B" jointly revised in 2010 by the Branch of Liver Disease and the Branch of Infectious Disease of the Chinese Medical Association. Cirrhosis and liver cancer were ruled out by B-scan ultrasonography. Patients with other viral hepatitis infections, HIV-positivity, and other autoimmune or infectious diseases were excluded.

Reagents and Equipment

HBV DNA was detected with a Roche LightCycler fluorescence quantitative PCR instrument (Indianapolis, IN, USA) with a lower limit of 500 IU/ml. HBsAg, HBeAg, and HBeAb were detected by electrochemiluminescence using a Roche Modular E170 system (Roche Diagnostics, Indianapolis, IN, USA). Liver function was analyzed with a Beckman Automatic Biochemical Analyzer (Beckman Coulter, Inc., Brea, CA, USA). The FACSCalibur flow cytometer was from Becton Dickinson (Franklin Lakes, NJ, USA). Human $CD8a^+$ T Cell Isolation Kit, Running Buffer, and LS Columns were from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). The FITC-labeled anti-human CD45RA antibody and phycoerythrin (PE)-labeled anti-human CCR7 antibody were from eBioscience Inc. (San Diego, CA, USA). Roswell Park Memorial Institute-1640 (RMPI-1640) medium and phosphate buffered saline (PBS) were from Corning Co. (Corning, NY, USA). The HBsAg proteins were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) IFN-γ ELISA kits were from R&D (Minneapolis, MN, USA).

Research Methods

After informing patients and receiving their consent, peripheral blood samples were used for the following experiments and tests.

Preparation of $CD8^+$ T lymphocytes from Peripheral Blood

Mononuclear cell suspensions were prepared aseptically. $CD8^+$ T lymphocytes were prepared using immunomagnetic beads, based on negative immunomagnetic separation, following the lysis of red blood cells using NH_4Cl, and immunoprecipitation of Roswell Park Memorial Institute (RPMI)-1640. Cells were resuspended at a concentration of 1×10^6/ml and tested for purity and viability. Samples were then stored at 4°C until use.

IFN-γ Detection

$CD8^+$ T cells were incubated with Interleukin-2 (IL-2) (final concentration of 10 ng/ml) and HBsAg (final concentration of 10 μg/ml) for 48 h. The culture supernatant was collected and stored at −20°C. The procedure was completed in strict accordance with the ELISA kit instructions. An ELISA instrument was used to measure the levels of IFN-γ secretion.

$CD8^+$ T cell detection

$CD8^+$ T cell populations were collected (roughly 5×10^5 per tube), resuspended in phosphate-buffered saline (PBS), centrifuged (1200 rpm, 5 min), and the supernatant was discarded. Appropriate amounts of PE-CD45RA and FITC-CCR7 antibodies were added, and well mixed with the $CD8^+$ T cells. The cells were incubated at 4°C for 30 min in the dark and washed three times with PBS. The samples were then subjected to flow cytometry, and the results were analyzed using Cellquest software (BD Biosciences, San Jose, CA, USA).

Statistical Analysis

SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Data are

Table I. General parameters of chronic hepatitis B patients.

Groups	No.	Age	Male	ALT (U/L)	HBVDNA (log_{10} IU/ml)
HBsAg seroconversion group	10	43.5 ± 14.3	6	25 ± 11	0
HBeAg group	25	39.4 ± 17.1	18	14 ± 5	0
CHB group	25	41.3 ± 13.2	17	95 ± 31	5.3 ± 1.4
HBV carrier group	25	31.5 ± 12.4	15	26 ± 12	6.5 ± 2.5

Table II. Percentages of CD8+ Subsets (%) in the chronic HBV infection patients.

Group	No.	TN (CD45RA+ CCR7+)	TCM (CD45RA- CCR7+)	TEM-1 (CD45RA-CCR7-)	TEM-2 (CD45RA+ CCR7-)
HBsAg seroconversion group	10	9.9 ± 4.5	1.4 ± 0.5	10.2 ± 3.3	83.5 ± 21.3
HBeAg group	25	22.5 ± 5.6[2]	1.2 ± 0.4	11.8 ± 3.5	65.5 ± 17.1[2]
CHB group	25	31.4 ± 8.2[1,4]	1.6 ± 0.6	13.3 ± 4.2	49.7 ± 13.4[1,4]
HBV carrier group	25	49.5 ± 11.3[1,3,5]	1.9 ± 0.8	14.0 ± 4.3	36.3 ± 8.2[1,3,6]

N: *vs*. HBsAg seroconversion group: 1: $p<0.01$; 2: $p<0.05$; *vs.* HBeAg seroconversion group; 3: $p<0.01$; 4: $p<0.05$; *vs.* CHB Group: 5: $p<0.01$; 6: $p<0.05$.

presented as mean ± standard deviation. Data were analyzed by variance analysis and *t*-test. $p<0.05$ was taken as statistically significant.

Results

General Information

The general parameters of patients in each group, HBV DNA, and alanine aminotransferase (ALT) are shown in Table I. There were no significant differences in sex or age between the groups ($p>0.05$).

Detection of CD8+ TM Subsets

There were no significant differences in the percentage of CD8+ TEM-1 memory T cells in peripheral blood between the different groups. The percentage of CD8+ TEM-2 memory T cells in peripheral blood was higher in the HBsAg seroconversion group than in the HBeAg seroconversion group ($p<0.05$) and CHB group ($p<0.01$), while the levels were lower than in the chronic HBV carrier group ($p<0.01$)

There were no significant differences between groups in the percentage of CD8+ TCM in peripheral blood. The percentage of CD8+ TN cells in peripheral blood was higher in the HBV carrier group than in the CHB group ($p<0.01$) and HBeAg seroconversion group ($p<0.01$), and lower in the HBsAg seroconversion group ($p<0.01$).

Levels of IFN-γ Secretion

The level of IFN-γ secreted by CD8+ T cells in peripheral blood was higher in the HBsAg seroconversion group than in the HBeAg seroconversion group ($p<0.01$) and CHB group ($p<0.05$), while they were lower in the HBV carrier group ($p<0.01$).

Discussion

In chronic HBV infection, because of immune tolerance of HBV, T cells, as the key immune cells necessary for clearing the virus, appear with dysfunction or incompetence. The antigen-presenting function of dendritic cells declines, and the cytokine network becomes disordered, resulting in difficulty of clearing HBV and a chronic infection. However, clinical observation has shown that some patients present with spontaneous elim-

Table III. The levels of IFN-γ secretion in the different groups.

	No.	IFN-γ (pg/ml)
HBsAgseroconversion group	10	632.5 ± 173.5
HBeAg group	25	499.4 ± 127.6[2]
CHB group	25	376.1 ± 105.7[1,3]
HBV carrier group	25	355.3 ± 91.4[1,3]

Note: *vs*. HBsAg seroconversion group: 1. $p<0.01$; 2. $p<0.05$; *vs*. HBeAg seroconversion group: 3. $p<0.05$.

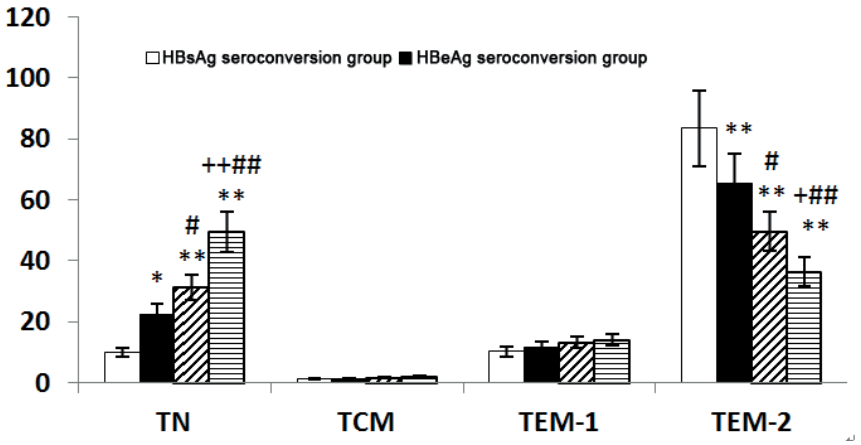

Figure 1. Percentage of $CD8^+$ Tm subsets of different outcomes of chronic HBV infection. *vs.* HBsAg seroconversion group: $^{**}p<0.01$; $^{*}p<0.05$; *vs.* HBeAg seroconversion group $^{\#\#}p<0.01$; $^{\#}p<0.05$; *vs.* CHB group: $^{++}p<0.01$; $^{+}p<0.05$.

ination of HBV, where HBV DNA in peripheral blood becomes negative, and HBeAg and HBsAg seroconversion occur, resulting in the remission of infection. It is generally believed that this is related to changes in the immune function of patients, although the specific immune mechanism is still unclear. T lymphocytes effector memory (TEM) is an important subset of memory T cells. TEM cells produce effector molecules such as IFN-γ, TNF-α, and perforin. Those effectors can kill infected cells through their cytotoxicity[6,7]. TEM includes TEM-1 cells and TEM-2 cells. In acute hepatitis B recovery, high levels of TEM-1 were observed with the removal of the virus[8]. Similar findings were also found in lymphocytic choriomeningitis virus infection, influenza virus infection, and other conditions. TEM-1 may be a key effector cell-type for viral clearance in acute infection. Similar to other reports, in the present study, $CD8^+$ TEM cells represented a high proportion of $CD8^+$ T cells in the peripheral blood of patients with chronic HBV infection, accounting for over 50%. However, the expression of $CD8^+$ TEM-1 cells was not significantly different between the groups, and the proportion was lower than 15%. $CD8^+$ TEM-1 cells were not significantly increased even with the spontaneous clearance of HBV. It is believed that $CD8^+$ memory T cells present with a disorder of differentiation, and may not be the key immune cells for the clearance of HBV. TEM2 cells arise from TEM cells[9], and express more perforins than TEM-1. They have a stronger chemotactic activity to β-chemokine and have a proliferative activity. In this study, the proportion of TEM-2 cells in patients with HBeAg or HBsAg spontaneous seroconversion was significantly higher (65% and 98%, respectively) than in chronic HBV carriers (34%) and patients with chronic hepatitis B (58%). It was suggested that increased TEM-2 cell differentiation may be beneficial for clearing HBV. Champagne et al[9] found that the differentiation defect of TEM-1 to TEM-2 can lead to the function of $CD8^+$ T-cell, or a homing defect. Hess et al[10] suggested that fully differentiated $CD8^+$ TEM-2 cells may have better potency than early memory cell populations, and found that $CD8^+$ TEM-2 cells were present in a large number of infected individuals that could control viral load. The percentage and absolute value of $CD8^+$ TEM-2 cells in slow progressors were significantly higher than in AIDS patients, and the percentage and absolute value of $CD8^+$ TEM-1 cells in slow progressors were significantly lower than in AIDS patients. Appay et al[11] found that TEM-2 cells with a $CD27^+CD28^-$ phenotype presented more during the recovery phase of acute viral infection and less in chronic viral infections. These observations further showed that highly differentiated $CD8^+$ TEM-2 cells may be beneficial for the spontaneous clearance of HBV, and the obstacle of differentiation to the TEM-2 subpopulation may lead to persistent HBV infection. In this study, we further demonstrated that the increase of IFN-γ secretion correlated with the ratio of $CD8^+$ TEM-2 cells. Higher levels of $CD8^+$ TEM-2 cells and IFN-γ were observed in patients with HBsAg or HBeAg seroconversion. We believe that the expression of $CD8^+$ TEM-2 cells can significantly increase the degree of the anti-HBV response in chronic infection. We also

found that $CD8^+$ TCM cells in peripheral blood were in low levels, and the proportions were less than 5% in each group. A possible cause was related to the obstacle of $CD8^+$ TCM differentiation[1,2]. Another possible cause was that the TCM homing receptor, CCR7, was mainly expressed in the secondary lymphoid tissue, and was expressed at low levels in peripheral blood. The proportion of $CD8^+$ TN cells ($CD8^+CDRA^+CCR7^+$) was significantly lower in the HBeAg and HBsAg seroconversion groups than in the control groups, which might have been related to the increased immune response of the patient to HBV, and the activation of TN to TEM.

Conclusions

We found that the significantly increased $CD8^+$ TEM-2 cell subset may be an important mechanism for the clearance of HBV (including the disappearance of HBV DNA and HBV-associated antigens). $CD8^+$ memory T cell subsets presented with a significant differentiation obstacle in chronic HBV infection compared with acute HBV infection. The specific role of $CD8^+$ memory T cells in chronic HBV infection requires further investigation because of the inability to further determine the immune function of $CD8^+$ TEM-2 cells in peripheral blood, and the number and function of $CD8^+$ memory T cell subsets in the liver. The detection of $CD8^+$ memory T cells in peripheral blood is beneficial for assessing the anti-HBV immune level of patients, guiding clinical treatment, and for the development of hepatitis B vaccines.

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Conflict of interest

The authors declare no conflicts of interest.

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