Original Article
Immunotherapy of patients with chronic hepatitis B virus infection by HBsAg-activated dendritic cells combining with cytokine induced killer cells

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Abstract: Purpose: Hepatitis B virus (HBV) is one of the most common human viruses threatening people's health and life. The objective of the present study was to explore clinical efficacy of HBsAg-activated dendritic cells (DCs) and cytokine induced killer (CIK) cells on patients with Chronic Hepatitis B (CHB). Methods: Fourteen patients suffering CHB who met the inclusion criteria were collected. Autologous DC and CIK cells were separately induced from peripheral blood mononuclear cells, and they were amplified and co-cultured. The obtained DC-CIK cells were used to treat CHB patients in immune therapy for 5 times. Clinical treating effects with autologous DC-CIK cells in patients with CHB were observed. Results: Comparing respective values before and after treatment, the change of HBsAg (P=0. 0.041) was statistically significant, but not the HBV-DNA level (P=0.975). After treatment, liver functions of patients were slightly changed without statistical significances such as TB (total bilirubin). In addition, the immune responses were not significantly activated since of CD3CD4, T helper\T suppressor cells and regulatory T cells. Conclusions: The clinical treatment effects of autologous DC-CIK on HBeAg positive CHB have not showed overall effectiveness in patients, which may be result of complicated patient characteristics including immune status. Therefore, further studies for the clinical efficacy of autologous DC-CIK for the treatment of CHB patients are still needed.

Keywords: Autologous DC-CIK, chronic hepatitis B, hepatitis B virus

Introduction

Hepatitis B virus (HBV) is one of the most common human viruses threatening people's health and life. In particular, some chronic hepatitis B (CHB) will lead to cirrhosis and hepatocellular carcinoma development in some patients [1]. According to WHO statistics, there are around two billion people are infected with HBV, 17.5% people of which are patients of CHB [2]. Nearly 300,000 people die of various diseases especially cancers caused by CHB infection each year in our country [3, 4].

Patients with CHB are in a state of high infection and low immune response stages in most circumstances, besides, HBeAg as one of the most important immune tolerance factors is vital in determining of them [5, 6]. Although presently available anti-HBV nucleoside drugs with strong and high inhibitory effects on HBV play important roles in the prevention and treatment of CHB, the serum conversion rate of HBeAg/anti-HBe is low [7]. Furthermore, repeated and long-term use of lamivudine leads to resistant mutants, resulting in ALT and HBV DNA breakthrough and contributing to disease progression [8]. Therefore, it is necessary and urgent to develop more effective, specific and lowest toxicity anti-HBV drugs both research and clinical applications.

Immune response-based therapy which is low or deficiency in patients with CHB [9] becomes a main direction via breaking states of immune tolerance, inducing effective cellular immunity, inhibiting and eliminating HBV in CHB patients [10]. Function of dendritic cells (DC) in patients with CHB is low or deficiency, leading to tolerance states of T and B lymphocyte responses

[11, 12]. Immune tolerance is one important obstacle to the treatment of CHB, and it is also one fact in maintaining virus infection [13]. Therefore, it is urgent to solve the bottleneck.

DC-CIK (cytokine-induced killer) biological treatment has been proved to be effective in the treatment of a broad spectrum of cancers through improving the immune functions and the anti-tumor immune reactions [14, 15]. Some study suggested that *in vitro* cultured targeted CIK can effectively compensate deficiency of CD8+CT (immune response) in with CHB patients [16]. Shi et al. suggested that cytokine-induced killer-cell treatment was only partially effective, not all 14 CHB patients they studied [17]. Analogously, Einsele et al. found that in some patients who received an intensified immune suppression at the time of or after T-cell therapy, only transient reductions in virus load were obtained [18]. Therefore, these defects may not fully correct after in vitro treatment. Though immunotherapy can be obtained more efficiently and turned out to be more specific in the killing of tumor cells [9], the total response rate was only 51.7% in them [19]. Therefore, the clinical efficacy of this immunotherapy for treatment of CHB needs further investigation in different clinical populations.

In the present study, autologous DC and CIK of patients with CHB were co-cultured in vitro, and the obtained DC-CIK cells were used to treat CHB patients with conventional approaches. Clinical treating effects and immune function in patients with CHB were observed, which will provide theoretical basis and may further clarify the availability for the new therapeutic method in the treatment of CHB.

Materials and methods

Subjects

All persons have given their informed consent prior to their inclusion in the study, and all human studies have been approved by our hospital Ethics Committee and performed in accordance with the ethical standards. Fourteen patients were infected with chronic HBV, who were HBsAg, HBeAg, and HBcAb-positive. There were 7 male cases and 7 female cases with ages of 15-48 years and mean age of 38 years. All subjects in the present study were adults.

Inclusion criteria

Patients with CHB were diagnosed according to the diagnostic criteria specified in "Chronic hepatitis B prevention and treatment guidelines" (2010 edition) [9]. The subjects were diagnosed as HBeAg-positive HBV infection with ages of 15-65 years. Parameters of liver function tests in patients were as the following levels: ALT < 400 U/ml, AST (aspartate aminotransferase) < 400U/ml, prothrombin activity > 50%, T-bil < 40 μmol/L, HCG + pregnancy test: negative, AFP < 50 ng/ml (Normal value: 0-7 ng/ml). For experiments involving human subjects, approval was obtained from the institutional review board of our hospital. Informed consent was provided according to the Declaration of Helsinki [12].

Exclusion criteria

The exclusion criteria were previously described [9, 11, 12] and the following people were excluded: pregnant and lactating women, people with multiple organs failure (including liver), people with organ transplants such as liver, heart and kidney, patients with severe autoimmune diseases such as SLE (Systemic Lupus Erythematosus), rheumatoid arthritis, or vasculitis, patients with infectious and uncontrollable diseases and the allergies patients to the biological reagent used in the present study.

Reagents

RPMI1640 medium, human lymphocyte separation medium (Ficol1) were purchased from Beijing Soledad Bao Technology Co. Ltd. Fetal bovine serum (FBS) was Hyclone product; CD3 monoclonal antibody was purchased from Wuhan Institute of biological technology company; IFN-r was purchased from Shanghai kaimao Biological Medicine Co Ltd; rhIL-2 was purchased from Liaoning biological product research satellite (limited company); rhGM-CSF, rhIL-1, TNF-a, rhIL-4 were products of Perprotech company; CCK-8 kit and IL-12 ELISA kit were purchased from Wuhan boster Biological Engineering Co., Ltd.; Hepatic cell line HepG2.2.15 was a kind gift from Professor Zheng Liyun of Zhengzhou University hepatology Institute.

Isolation, cell culture, amplification and induction of DC and CIK cells

Isolation, cell culture, amplification and induction of DC and CIK cells followed as described

Table 1. Clinical indicators after HBsAg sensitized DC-CIK treatment

	Before treatment	After treatment	P-value
Virus load			
HBsAg	17447.95 (6859.725-25000)	14631.7 (2569.1-25000)	0.041
HBeAg	790.11 (9.92-1071)	646.01 (28.56-1097.75)	0.177
HBV (DNA)	60500000 (3250000-173000000)	51000000 (1070000-198000000)	0.975
Blood routine			
WBC*10^9	5.58±0.41	5.90±0.43	0.461
NC*10^9	3.44±0.47	3.52±0.33	0.874
PLT	160.5 (113.5-213.75)	176.5 (129.25-195.75)	0.572
Liver function			
TB	12.71±3.94	13.36±1.23	0.386
ALT	59.7 (28.875-85.35)	49.05 (20.275-76.975)	0.346
AST	40.1 (23.475-61.5)	29.3 (22.75-72.5)	0.33
A/G	1.58±0.17	1.57±0.22	0.934
GGT	35.5 (22.75-62.275)	31 (21.725-60.85)	0.510
Immune response			
CD3	49.76±5.40	52.54±3.92	0.491
CD3CD4	55.3 (52.075-64.7)	61.15 (47.15-69.475)	0.530
CD3CD8	34.75±1.77	34.91±2.55	0.902
TH/TS	1.75 (1.4-2.125)	2.05 (1.138-2.6)	0.614
NK	12.95 (9.775-15.075)	15.05 (6.725-18.125)	0.875
NKT	5.5 (2.75-10.25)	2 (1-6)	0.275
Treg	1.77 (0.7725-2.48)	1.885 (0.4225-3.855)	0.272

previously [20, 21]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from blood of CHB patients with human lymphocyte separation medium. After two hours incubation at 37°C, non-adherent cells were collected and IFN-r was used. A 24 hours followed cultivation was conducted. Then the CD3 monoclonal antibody and rhIL-2 were added. CIK medium was changed or added with the proliferation of cells. Cell densities were determined regularly. For the DCs induction, adherent cells were collected; rhGM-CSF and rhIL-4 were cultured for 5 days. At the sixth day, rhIL-1 and TNF-a were also added to maturate of DC. The harvested DC and CIK cells were co-cultured at a ratio of 1:50-100.

Study procedure

From June 2010, patients with CHB who met the inclusion criteria were collected. Samples of the patients were obtained before treating them with various tests including hepatitis B surface antigen, E antigen, HBV DNA, liver function, blood routine and CD3CD4, CD3CD8, TH/TS (T helper\T suppressor cells), NK (Natural Killer), NKT (natural killer T), Treg (Regulatory T cells).

Peripheral blood (hematopoietic mobilization with GM-CSF before blood sampling) with HBeAg-positive in patients with CHB was harvested to obtain mononuclear cells. DC cells and CIK cells were generated by inducing lymphocytes in vitro. These cells were co-cultured to provide DC-CIK cells. DC-CIK cells co-cultured for 48 h were collected for the detection of cytokines using the method of ELISA. Each sample of cytokines was detected of A450 value according to previous studies [20-22].

Patients were given intravenous input of the prepared cells every week. Five million cells were transfused each time with a total of 5 times.

After one month of the last treatment, specimens from patients were kept and were conducted with various tests including laboratory hepatitis B surface antigen, E antigen, HBV DNA, liver function, blood routine, CD3CD4, CD3CD8, TH/TS, NK, NKT and Treg.

Statistical methods

SPSS 13.0 statistical software was used in statistical analysis. The Shapiro-Wilks test was applied for normality test and data with *p*-value more than 0.05 is considered to be normally distributed. Indicators those normally distributed both before and after treatment were presented by its mean and standard deviation, if not, they were presented by median and interquartile range (IQR). The difference between two groups was compared by the unpaired t test (normal distribution) or the Wilcoxon signed rank test (non- normal distribution). The two-tailed P < 0.05 indicated that the difference was statistically significant.

Results

Effects of DC-CIK treatment on virus parameters

Effects of DC-CIK treatment on patients with CHB were investigated by ELISA in the present study firstly. As demonstrated in **Table 1**, changes of hepatitis B surface antigen, E antigen and HBV DNA were observed. Comparing respective values before and after treatment, HBsAg (P=0.041) was statistically significant. The HBV-DNA load of post treatment was similar to that before treatment (P=0.975).

Effects of DC-CIK treatment on liver functions

Changes of TB (total bilirubin), AIL (alanine aminotransferase), AST, A/G (albumin/globulin ratio), GGT (glutamyl transpeptidase) before and after treatment were explored in the presents study in order to understand effects of DC-CIK treatment on liver function. TB, A/G and GGT shown no statistical difference with respective *p*-values were 0.386, 0.934 and 0.510.

Effects of DC-CIK treatment on immune responses

Changes of DC-CIK related CD3CD4, CD3CD8, TH/TS, NK, NKT, Treg before and after treatment were studied and the results were illustrated in **Table 1**. There were no significant between these two groups, including CD3CD4 (P=0.530), TH/TS (P=0.614), NKT (P=0.275) and Treg (P=0.272).

Discussion

HBV is one of the most common human viruses threatening people's health and life [23]. Co-mbined immunotherapy with DC and CIK cells seems to show positive impact on the treatment of CHB. However, clinical treatment effectiveness of autologous DC-CIK on HBeAg positive chronic hepatitis B remains further investigation.

HBsAg has recently been proposed to guide treatment response of CHB. HBsAg seroconversion defined by the loss of serum HBsAg and the increase of anti-HBs antibodies is the denotation of a successful immunological treatment of HBV infection [24]. In our study, HBsAg level was decreased in the group after treatment when compared to the value measured before treatment. This may mean the HBsAg seroconversion process promoted by the immunotherapy [24]. Brunetto et al. proposed that HBsAg of which expression level did not change after DC-CIK cell infusion in the treatment and control groups, does not associated with a complete immune response in CHB patients [25].

Though the HBV DNA level had the declining trend in patients after treatment, however, there were great differences among 14 patients not only before but also after treatment, thus this may be the reason no statistical significance between them. In addition, there was no significant difference about the indicators of HBV load, liver function or immune response examined pre-therapy and post-therapy in all patients during our study. Two similar studies performed by Shi et al. showed that autologous CIK cells which was more effective in some patients was an alternative immune therapeutic strategy for CHB [17, 26]. In our current study, though some indicators in particular patients indicated great differences before and after treatment (data not shown), the total efficacy of immune response and virus load showed no statistical differences. Therefore, the efficacy of DC-CIK cell treatment for CHB patients may be mainly dependent on the patient characters including immune status [26].

Conclusions

In conclusion, during this study, the obtained DC-CIK co-cultured cells were used to treat CHB patients in immune therapy. Comparison of immune response, liver function and virus load between before and after treatment did not show the potential role of autologous DC-CIK on HBeAg positive CHB. This may be

due to the great differences among individuals. Therefore, autologous DC-CIK is not a widely used therapy for all patients, at least the CHB patients. There still some limitations of this article, such as the lack of control experiment and the samples size were not enough. Especially, it is currently a personalized therapeutic approach, large prospective randomized trials are still difficult. Therefore, further studies were still needed to clarify this.

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Dislosure of conflict of interest

None.

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