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Changes in peripheral blood natural killer T cells in hepatitis B e antigen-positive chronic hepatitis B patients and efficacy prediction after pegylated interferon therapy

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ABSTRACT. We examined the expression of peripheral blood natural killer T (NKT) cells in hepatitis B e antigen (HBeAg)-positive chronic hepatitis B (CHB) patients and predicted its efficacy after pegylated interferon α-2a (Peg-INFα-2a) therapy. Sixty-three cases of HbeAg-positive CHB inpatients and outpatients, treated in the Third Xiangya Hospital of Central South University from January to December 2010, were administrated Peg-INFα-2a 18 myriad international unit intramuscularly once per week for 48 weeks. The number of peripheral NKT cells, 5 quantitative indicators of hepatitis B, and hepatitis B virus DNA capacity were detected at each time point. Forty-eight weeks after Peg-INFα-2a treatment, 26 HBeAg-positive CHB patients exhibited significant effects,

21 cases exhibited effects, and 16 cases showed no effects. The ratio of peripheral blood NKT cells in T lymphocytes before and 4, 8, and 12 weeks after treatment in the significant effect group was significantly increased compared to the effect group and no effect group ($P < 0.01$); at the 48th week of treatment and 24 weeks after the drug was withdrawn, NKT cell expression in the significant effect group was significantly higher than that in the effect group ($t = 32.0$, $P < 0.01$; $t = 27.6$, $P < 0.01$, respectively). A total of 27 patients showed HBeAg seroconversion until the 24th week after drug withdrawal. During treatment with Peg-INFα-2a in HBeAg-positive CHB patients, expression of peripheral blood NKT cells could be used to predict efficacy.

Key words: B-type hepatitis; Hepatitis virus; Hepatitis B e antigen; Pegylated interferon α-2a; Virologic response

INTRODUCTION

Chronic hepatitis B (CHB) is a chronic infectious disease caused by hepatitis B virus (HBV)-induced immune clearance, which causes hepatocyte damage (Lok and McMahon, 2009). The unclear immune clearance makes CHB treatment difficult (Han et al., 2013); the key to effective CHB treatment is combination therapy involving immune regulation and direct antiviral treatment (Tang et al., 2005). Pegylated interferon (Peg-INF) is internationally recognized as an effective first-line antiviral drug for treating CHB (Abaalkhail et al., 2014). It not only has direct antiviral activity, but also regulates the immune system by activating immune cells to clear the virus (Koktekir et al., 2013). There is no reliable method for predicting early efficacy, and thus subsequent treatment programs cannot be determined early. Natural killer T (NKT) cells are recently identified lymphocytes that largely exist inside the liver and regulate the body's immune response with high efficiency (Bendelac et al., 2007; Gao et al., 2009). Its antiviral activities toward HBV infection have been demonstrated in previous studies (Kakimi et al., 2000; Zeissig et al., 2012). In our previous studies, we found that the peripheral NKT expression level in the active phase of the immune response was significantly higher than that during the immune tolerance phase (Huang, 2007; Jiang et al., 2011). Therefore, further observation was performed to determine whether Peg-INF therapy could regulate the body's immune cells and activate the *in vivo* expression of natural killer (NK)/NKT cells, thus improving antiviral activities. We examined Peg-INF therapy for treating CHB, determined the expression of NKT cells in patients, and examined the relevance of their immune activities and effects of antiviral therapy to further understand the relevance of NKT cells and CHB immune tolerance, as well as analyzed changes in NKT cells towards the efficacy prediction of IFN antivirus therapy.

MATERIAL AND METHODS

Subjects

Sixty-three hepatitis B e antigen (HBeAg)-positive CHB inpatients and outpatients, who were treated with Peg-INFα-2a (Pegasys, Shanghai Roche Pharmaceutical Co. Ltd., Shanghai, China) in the Third Xiangya Hospital of Central South University from January to December

2011, were selected, including 41 males and 22 females, aged 18-55 years with a mean age of 39 years, and disease duration of 8.12 ± 3.68 years. The patients voluntarily accepted Peg-INF antiviral therapy and met the following conditions: 1) HBeAg (+), 2 x 10^4 IU/mL ≤ HBV DNA < 2 x 10^7 IU/mL; 2) 2X upper limits of normal (ULN) ≤ alanine aminotransferase < 10X ULN, serum total bilirubin < 2X ULN; prothrombin activity was essentially normal; 3) did not use antiviral drugs and immune modulators 6 months before treatment, and excluded other viral or systemic diseases; 4) without absolute and relative contraindications of IFN therapy; 5) during treatment, neutrophil count was <0.5 x 10^9/L and/or platelet count was <30 x 10^9/L, total bilirubin was >51 mM (particularly in those who mainly had indirect bilirubin), would be symptomatically treated and withdrawn the drug; 6) could perform regular follow-up, with effective treatment and continued through the 48th week, followed by 24-week follow-up after withdrawing the drug. The patients who exhibited no effects after 24-week medication, then withdrew or switched to the nucleoside drug treatment, were not included. All patients met the diagnosis criteria of "Guideline of Viral Hepatitis Prevention", 2012 edition (European Association for Study of Liver, 2012). This study was conducted in accordance with the Declaration of Helsinki and was conducted with approval from the Ethics Committee of Central-South University. Written informed consent was obtained from all participants.

Medication

Peg-INFα-2a 18 myriad international unit was subcutaneously injected once per week for 48 weeks. Enzyme-reducing drugs were not used during the study period.

Observation indicators

The ratios of peripheral NKT cells towards peripheral T lymphocytes were detected before and 4, 8, 12, 16, 24, and 48 weeks after treatment. Five items were quantitatively measured in serum hepatitis B 5, and HBV DNA loading was detected at the 4th, 12th, 16th, 24th, and 48th week after treatment. The patients' blood, liver function, blood sugar, hyperthyroidism, and urine were monitored according to the 2012 guidelines, and the patient's mental state was also periodically assessed.

Detection method

Immunofluorescence real-time quantitative polymerase chain reaction was used to detect HBV DNA, with a detection limit of 500 IU/mL for NKT cell detection. Next, 100 μL ethylenediaminetetraacetic acid-anticoagulated venous whole blood was added to 20 μL fluorescein isothiocyanate anti-human NKG2D and 10 μL PE-Cy5 anti-human CD3 (BD Biosciences, Franklin Lakes, NJ, USA), then shaken and incubated in the dark at room temperature for 30 min; 2 mL erythrocyte lysate was then added, shaken, and incubated. The supernatant was discarded and the residue was washed with phosphate-buffered saline and mixed with 220 μL 1% paraformaldehyde fixation solution, incubated, and subjected to detection by flow cytometry in the dark.

Efficacy determination criteria

For the complete virologic response (significantly effective, SE), HBV DNA was negative, peripheral HBV DNA was <500 IU/mL, with HBeAg becoming negative or seroconver-

ting, and alanine aminotransferase was normal. For the partial virologic response (effective, EF), HBV DNA was under control, serum HBV DNA decreased to >2 log10 IU/mL compared with the baseline, with or without HBeAg seroconversion, and alanine aminotransferase may be normal. For the no virologic response (invalid, IV), serum HBV DNA decreased to <2 log10 IU/mL compared to baseline and no HBeAg seroconversion was observed.

Statistical analysis

The SPSS13.0 statistical package was used for statistical analysis (SPSS, Inc.; Chicago, IL, USA). The counting results of flow cytometry are reported as means ± standard deviation. For the comparison of mean NKT among the SE group, EF group, and IV group within 0-24 weeks, we used the SNK-q test of variance analysis; for comparison of the mean NKT between the 48th-week and 24-week drug withdrawal group, we used the Student *t*-test.

RESULTS

Effects of Peg-INFα-2a treatment

After 48-week Peg-INFα-2a treatment, 26 cases showed SE, 21 cases exhibited EF results, and 16 cases were IV.

NKT cells

Flow cytometry was used to detect the ratio of peripheral NKT cells in peripheral T lymphocytes (Table 1). The expression level of NKT in the SE group was significantly increased compared to that in the EF group and the IV group at all of the time points, and the differences were statistically significant ($P < 0.01$); at 48th-week medication and 24th-drug withdrawal, the NKT expression levels in the SE group were significantly increased compared to those in the EF group and the IV group ($t = 32.0$, $P < 0.01$; $t = 27.6$, $P < 0.01$, respectively). In the first 4 weeks of medication, NKT expression levels in the SE group increased rapidly, reaching a peak at the 12th week, then gradually decreased from the 24th week to a slightly higher level than that before treatment; this level was maintained until the 48th week. The NKT level in the EF group peaked at the 12th week, which was significantly higher than that before treatment ($t = 12.83$, $P < 0.05$).

Table 1. Ratio of NKT in the blood of each group (%).

Time	Group				
	SE group (N = 26)	EF group (N = 21)	IV group (N = 16)	*F/t*	P
0 week	26.26 ± 2.90	20.22 ± 1.73	13.17 ± 0.94	133.13	<0.01
4th week	39.19 ± 3.28	25.22 ± 2.88	15.71 ± 1.02	264.55	<0.01
8th week	42.45 ± 3.11	26.58 ± 3.12	16.18 ± 1.72	409.15	<0.01
12th week	43.11 ± 5.75	27.16 ± 0.94	16.93 ± 2.01	436.24	<0.01
16th week	35.15 ± 5.36	25.35 ± 1.25	14.69 ± 2.05	295.87	<0.01
24th week	29.98 ± 8.78	21.02 ± 3.21	13.12 ± 1.32	111.80	<0.01
48th week	30.12 ± 4.43	22.53 ± 4.15		*t* = 32.0	<0.01
24 weeks after withdrawal	27.08 ± 1.25	21.36 ± 2.31		*t* = 27.6	<0.01

SNK-q test revealed that the pairwise comparison among the 3 groups was statistically significant, $P < 0.05$ (i.e., comparison between SE and EF, comparison between SE and IV, comparison between EF and IV, all $P < 0.05$, the Student *t*-test was used to compare SE and EF). SE = significantly effective; EF = effective; IV = invalid.

Comparisons of the mean NKT levels in the 3 groups at all time points from 0 to 24 weeks revealed that expression in the SE group was significantly higher than in the EF group and IV group (all $P < 0.01$); at the 48th week of medication and 24th week after drug withdrawal, mean NKT levels in the SE group were significantly higher than in the EF group (all $P < 0.01$).

Liver function and HBV DNA

Liver function was monitored in all patients at each time point. Patients in the SE group completely returned to normal at approximately 12 weeks, and continuously maintained normal levels, while relative HBV DNA load gradually decreased. At the 24th week, 22 patients in the SE group exhibited HBeAg seroconversion, while liver function in the EF group and IV group fluctuated at approximately 1-2X ULN. Until the 48th week, 3 patients in the EF group showed HBeAg seroconversion. Monitoring was performed until the 24th week after drug withdrawal, and 27 cases showed HBeAg seroconversion (Table 2).

Table 2. All patients' liver function and quantitation of HBV DNA, and HBeAg seroconversion in each stage.

Time	ALT (U/L)	HBV DNA (IU/mL)	HBeAg seroconversion (Case)
0 week	146 ± 42	$(8.58 \pm 2.42) \times 10^6$	0
4th week	58 ± 23	$(5.93 \pm 2.54) \times 10^5$	18
8th week	43 ± 28	$(6.22 \pm 2.75) \times 10^4$	22
12th week	35 ± 12	$(1.53 \pm 0.56) \times 10^4$	23
16th week	44 ± 8	$(2.61 \pm 0.89) \times 10^3$	23
24th week	34 ± 12	$(2.51 \pm 1.32) \times 10^3$	23
48th week	31 ± 15	$(1.84 \pm 1.03) \times 10^3$	25
24 weeks after withdrawal	28 ± 17	$(2.57 \pm 2.64) \times 10^3$	27

DISCUSSION

The NKT cells account for approximately 5% of T cells in the peripheral blood or spleen, and more than 50% in the bone marrow and liver (Emoto and Kaufmann, 2003). NKT cells can recognize the CD1d antigen complex, become activated, and function in innate immunity to cause an adaptive immune response (Zimmer et al., 2006). The cytokines secreted by NKT cells, such as IFN-γ, and thus activate NK cells and CD8+ T cells, all play inhibitory roles against viral replication (Biron et al., 1999). When HBV infection occurs, NKT cells, which are largely present in the liver, accept the CD1+ cell-presented lipid antigen and become activated (Baron et al., 2002). These lipid antigens are derived from the glycolipids and phospholipids of HBV subviral particles. Under the roles of virus-induced cytokines, NKT cells can be activated indirectly (Wang et al., 2013). The anti-HBV roles of NKT cells are mainly expressed in 2 aspects (Li et al., 2011), including through its secreted cytokines and by activating other lymphocytes. The interferon can be expressed as the viral marker (antigen), thus making recognition by the immune system easier; however, interferon may also directly promote the activities of NK cells, macrophages, and T cells, enhancing their killing functions (Bingfa et al., 2009; Senturk et al., 2011). NKT cells secrete IFN-γ, which may synergize and enhance antiviral activities. We found that 63 CHB patients receiving interferon antiviral therapy exhibited significantly different NKT expression levels. NKT expression levels in the SE group were significantly higher than those in the IV group ($P < 0.01$), and higher than those in the EF group. During the course of antiviral treatment, NKT expression levels gradually

increased. In the first 4 weeks, NKT levels increased very rapidly, reaching a peak at the 12th week, then gradually decreased until the levels were slightly higher than those before treatment at the 24th week. These levels were maintained until the 48th week, and 24 weeks after drug withdrawal, follow-up revealed that they were still higher than before treatment; however, the differences were not statistically significant. Peripheral NKT expression levels in the EF group were all significantly higher than those in the IV group before and after treatment ($P < 0.05$), and at the 24th week of treatment, liver functions had returned to normal levels. The 24-week follow-up revealed that 27 patients showed seroconversion from HBeAg to HBeAb and maintained the sustained virologic response, which is consistent with the high expression level of NKT.

NKT cell-secreted IFN-γ played a major role in the process of anti-HBV infection. NK cells were activated by NKT cells in an IFN-γ-dependent manner, thus acting as an anti-virus. Activation of NK/NKT cells may be important for resolving immune tolerance and clearing HBV (Godfrey et al., 2012). In this study, we found that detection of NKT expression levels could be used to predict the effects of interferon antiviral treatment, as well as predict the probabilities of virologic response or seroconversion. At the 24th week of medication, the NKT expression level peaked and then gradually declined, while maintaining higher levels until 48 weeks of medication and 24 weeks after drug withdrawal. Additional cases should be examined, and an NKT activator should be used to explore whether this significantly increases antiviral effects. This may enable NKT cells to secrete the IFN-γ to fight against HBV, possibly by blocking its subsequent inflammatory processes (e.g., block the chemokine ligand 9 and functions), which may be useful for treating viral hepatitis (Kakimi et al., 2001). Furthermore, the dendritic cell-secreted interleukin-12 may activate NKT cells, causing selective activation of the immune response by T helper cells. Accordingly, NKT systems can be targeted by anti-infection vaccines. The vaccine's activation roles towards NKT cells would activate a pathogen-specific immune response, and may also be included in new anti-virus programs (Taniguchi et al., 2003).

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