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BRIEF ARTICLE

Pegylated interferon α-2b up-regulates specific CD8+ T cells in patients with chronic hepatitis B

Ji Chen, Yan Wang, Xue-Jie Wu, Jun Li, Feng-Qin Hou, Gui-Qiang Wang

Ji Chen, Yan Wang, Xue-Jie Wu, Jun Li, Feng-Qin Hou, Gui-Qiang Wang, Department of Infectious Diseases, Center for Liver Diseases, Peking University First Hospital, Beijing 100034, China

Author contributions: Chen J and Wang Y contributed equally to this work; Chen J performed the laboratory work and wrote the paper; Wang Y collected the clinical samples and data, joined part in writing; Wu XJ performed the laboratory work; Li J and Hou FQ collected the samples and the data; Wang GQ designed the project and prepared the writing and supported all the work.

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Correspondence to: Gui-Qiang Wang, MD, Professor of Medicine, Chairman of Department of Infectious Diseases, Center for Liver Diseases, Peking University First Hospital, No. 8, Xishiku Street, Xicheng District, Beijing 10034, China. wanggq@hotmail.com
Telephone: +86-10-66551122 Fax: +86-10-66551680

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Abstract

AIM: To investigate the effect of pegylated interferon (IFN) α-2b on specific CD8+ T lymphocytes in patients with chronic hepatitis B (CHB).

METHODS: Twenty-one patients with CHB were treated with pegylated IFN α-2b. Periphery blood mononuclear cells were isolated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation (density: 1.077 g/L, Pharmingen) at weeks 0, 4, 8, 12, and 24, respectively. Frequency of circulating hepatitis B virus (HBV) epitope-specific CD8 T cells was detected by flow cytometry. Cytokines were detected by cytometric bead assay.

RESULTS: The frequency of circulating HBV core or env-specific CD8 T cells was higher ($P < 0.05$), the number of HBV core specific CD8 T cells was greater at week 24 ($P < 0.05$), the level of Th1-type cytokines [interleukin (IL)-12, tumor necrosis factor-α, and IFN-γ] was higher, while that of Th2-type cytokines (IL-4, IL-6, and IL-10) was lower in responders than in non-responders ($P < 0.05$) after pegylated IFN α-2b treatment. The IL-6 level was correlated with HBV DNA ($r = 0.597$, $P = 0.04$), while the inducible protein-10 (IP-10) level was correlated with serum alanine aminotransferase (ALT) ($r = 0.545$, $P = 0.005$). The IP-10 level at week 8 after pegylated IFN α-2b treatment could predict the normalization of ALT in CHB patients (positive predict value = 56%, negative predict value = 92%).

CONCLUSION: Pegylated IFN α-2b can enhance the immune response of CHB patients by increasing the frequency of HBV specific CD8+ T cells and regulating the Th1/Th2 cytokines.

Key words: Chronic hepatitis B; Pegylated interferon α-2b therapy; Immune response; Cytokine

Peer reviewer: Dr. Jeff Butterworth, MB, FRCP, Department of Gastroenterology, Shrewsbury and Telford Hospital NHS Trust, Mytton Oak Road, Shrewsbury, Shropshire, SY3 8XQ, United Kingdom

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INTRODUCTION

More than two billion people have been infected with hepatitis B virus (HBV) and chronic HBV infection affects about 400 million people worldwide[1,2]. Chronic hepatitis B (CHB) is a chronic inflammatory liver disease,

which can progress to end-stage liver diseases, such as cirrhosis and hepatocellular carcinoma.

Adaptive immunity plays a central role in the pathogenesis of chronic HBV infection, and it is crucial to understanding the behavior of T cell response for the design of effective strategies for the control of HBV infection[3-5]. Different studies in chronic and early acute phases of HBV infection suggested that the functional impairment of HBV-specific cell-mediated immune response plays an important role in HBV persistence[6-14]. Moreover, recent studies showed that both positive and negative signals regulate the antigen-specific T cell function and are important for the better outcome of patients with HBV infections[15-17].

Pegylated interferon (IFN) α-2b can modulate and reduce antiviral function of CHB patients by enhancing their immune responses. However, the exact effect of pegylated IFN α-2b on the immune responses of patients with HBV infections remains unclear. The present study was designed to investigate the effect of pegylated IFN α-2b on HBV specific CD8+ T cells and secretion of cytokines in CHB patients.

MATERIALS AND METHODS

Patients and study design

Twenty-one consecutive CHB patients (17 males and 4 females) at the age of 20-39 years (mean 25 years), admitted to our hospital from January 2008 to May 2009 were included in this study. Diagnosis of HBV infection was established as previously described[18]. Clinical data and characteristics of the patients are summarized in Table 1. The patients were treated with pegylated IFN α-2b (PegIntron from Schering-Plough), at the dose of 0.5-1 μg/kg of body weight, once a week for 24 wk. Clinical and laboratory data about the patients were detected before treatment, or at weeks 4, 8, 12, and 24 after treatment. Patients co-infected with HBV and HCV or with detectable antibodies against hepatitis delta virus or against human immunodeficiency virus were excluded, as were those with other causes of liver disease, including alcohol abuse. No patient had decompensated liver disease (evidence or history of ascites, variceal bleeding, hepatic encephalopathy or jaundice).

Isolation of peripheral blood monouclear cells

Peripheral blood monouclear cells (PBMC) were isolated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation (density: 1.077 g/L, Pharmingen). Blood was two-fold diluted with RPMI 1640 medium containing 300 μg/mL L-glutamin, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal calf serum, then added into the isovolumic Ficoll, centrifuged for 400 × *g* at 21℃ for 35 min. The cells were washed twice with phosphate buffered saline (PBS).

Human leukocyte antigen-A2 typing

One hundred microliters of fresh heparinized blood (100) was incubated with human leukocyte antigen-A2 primary antibody for 30 min. Erythrocytes were lysed with an erythrocyte lysate at 37℃, washed with PBS, and then incubated with secondary antibody, washed again and analyzed on Becton Dickinson FACS (Becton Dickinson, USA).

Analysis of HBV epitope-specific CD8+ T cells

Frequency of HBV epitope-specific CD8 T cells was detected by flow cytometry after incubated with HBV core18-27 tetramers (ProImmune, Oxford, UK) and HBV env 335-343 pentamers (ProImmune, Oxford, UK). Freshly isolated PBMC were incubated with PE-labeled tetramer or pentamer in PBS (10% FCS) for 15 min at 37℃, washed once with PBS (1% FCS) and then incubated on ice for 30 min with FITC-anti-CD8 (ProImmune, Oxford, UK), washed twice with PBS, adjusted to 1×10^6 cells/vial, and fixed in 2% paraformaldehyde for analysis. About 1×10^6 PBMC were harvested and analyzed within the CD8 gate on Becton Dickinson FACS using the CELLQuest™ software.

Secretion of cytokines

Serum levels of interleukin (IL)-2, IL-4, IL-5, IL-6, IL-10, IL-12, IFN-γ and inducible protein-10 (IP-10) in CHB patients were measured by cytometric bead assay (BD, USA) according to its manufacturer's instructions.

Serological assessment

Fasting serum levels of liver enzymes [alanine aminotransferase (ALT), aspartate aminotransferase] were measured with a Hitachi-7180 automatic biochemistry analyzer (Hitachi Inc., Japan) following the standard laboratory methods. HBV DNA was detected by real time polymerase chain reaction (Amplicor, Roche).

Statistical analysis

All data were analyzed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). Descriptive baseline data were expressed as mean ± SD for continuous variables. Differences between groups were assessed using Kruskal-Wallis H for continuous variables. Spearman *P* test was performed for correlation analysis. The accuracy of serum factors for predicting virologic response was assessed using the receiver operating characteristic curve. $P < 0.05$ was considered statistically significant.

RESULTS

Frequency of circulating HBV epitope-specific CD8 T cells in CHB patients after pegylated IFN α-2b treatment

Circulating HBV epitope-specific CD8 T cells were detected 13 out of the 21 CHB patients (Table 1). The frequency of HBV core 18-27 tetramers+/CD8+ T cells at week 0 was 0.013 ± 0.002, which increased to 0.026 ± 0.015, 0.029 ± 0.019, 0.036 ± 0.025, and 0.045 ± 0.027, respectively, at weeks 4, 8, 12, and 24 after IFN α-2b treatment (Figure 1), with a significant difference between weeks 8 and 0, and between weeks 24 and 0 ($P < 0.05$). The frequency of HBV env 335-343 pentamers+/CD8+ T cells began to increase at week 8 with a significant difference between weeks 24 and 0 ($P < 0.05$). No significant difference was observed in frequency of HBV core and HBV env specific CD8 T cells.

Table 1 Clinical characteristics of chronic hepatitis B patients included in this study

Patient	Age (yr)/sex	HBV DNA (IU/L)	ALT (U/L)	Total bilirubin (mg/dL)	Albumin (g/dL)	Platelets ($\times 10^9$/L)	HBeAg	HBeAb	HBsAg	HBsAb	Genetype
1	27/M	201 000 000	143	9.1	43.7	113	+	-	+	-	C
2	25/M	160 000 000	147	12.9	47.4	167	+	+	+	-	C
3	30/M	471 000 000	205	11.9	44.3	127	+	-	+	-	C
4	21/M	32 200 000	123	10.8	45.8	181	+	-	+	-	C
5	21/M	186 000 000	148	14.2	44.1	110	+	-	+	-	C
6	38/F	30 800 000	347	18.1	45.5	126	+	-	+	-	C
7	20/F	29 000 000	171	10.0	44.9	284	+	-	+	-	C
8	20/M	143 000 000	-	13.6	48.0	248	+	-	+	-	B
9	20/F	621 000 000	112	13.4	48.5	170	+	-	+	-	C
10	23/M	597 000 000	196	18.9	43.2	201	-	+	+	-	C
11	20/F	2 910 000	98	15.6	48.0	137	+	+	+	-	C
12	38/M	63 700 000	206	11.1	51.2	142	+	+	+	-	C
13	28/M	134 000 000	138	9.0	45.9	174	+	+	+	-	C
14	25/M	237 000 000	93	16.9	51.3	130	+	-	+	-	B
15	39/M	1 190 000 000	122	18.2	50.6	166	+	-	+	-	C
16	36/M	8 820 000	170	22.9	47.1	169	+	-	+	-	C
17	25/M	655 000 000	90	20.9	46.8	161	+	-	+	-	B
18	25/M	157 000 000	164	24.3	47.3	209	-	+	+	-	C
19	20/M	290 000 000	124	11.9	47.8	140	+	-	+	-	B
20	23/M	655 000 000		19.9	44.6	194	+	-	+	-	B
21	28/M	15 300 000	237	14.5	45.3	154	+	-	+	-	C

HBV: Hepatitis B virus; ALT: Alanine aminotransferase; HBeAg: Hepatitis B e antigen; HBeAb: Hepatitis B e antibody; HBsAg: Hepatitis B surface antigen; HBsAb: Hepatitis B surface antibody.

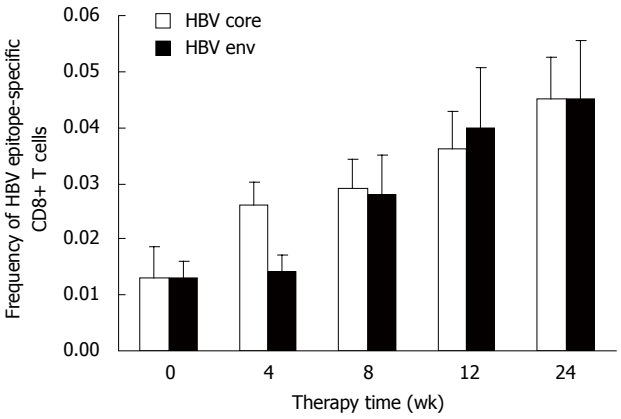

Figure 1 Frequency of hepatitis B virus epitope tetramer+/CD8+ T cell after pegylated interferon α-2b treatment. The frequency of hepatitis B virus (HBV) specific CD8+ T cells was increased connectively at weeks 4, 8, 12 and 24 after pegylated interferon α-2b treatment with no difference in frequency of HBV core specific CD8+ T cells and HBV env specific T cells.

To further analyze the effect of pegylated IFN α-2b on HBV-specific CD8 T cells, 13 patients were divided into responders (n = 7) and non-responders (n = 6). Responders were defined as their ALT returned to its normal level and their HBV DNA was decreased to over 2log, and/or their serum HBeAg was conversed. The frequency of HBV core18-27 tetramers+/CD8+ T cells was 0.014 ± 0.011, 0.029 ± 0.022, 0.029 ± 0.021, 0.067 ± 0.029, and 0.05 ± 0.025, respectively, in responders at weeks 0, 4, 8, 12 and 24 after treatment, which was higher than that in non-responders (0.012 ± 0.007, 0.018 ± 0.009, 0.028 ± 0.019, 0.025 ± 0.021 and 0.030 ± 0.01, respectively). No significant difference was found in frequency of HBV core specific CD8 T cells between responders and non-responders at baseline, even at weeks 4, 8, and 12 after treatment (Figure 2), with a significant difference observed at week 24 ($P < 0.05$, Figure 3). The frequency of HBV env specific CD8 T cells was higher in responders than in non-responders ($P < 0.05$, Figure 2).

Secretion of cytokines after pegylated IFN treatment and its correlation with virologic responses

The serum levels of IL-2, IL-4, IL-6, IL-10, tumor necrosis factor (TNF)-α, IFN-γ, IL-12, and IP-10 were measured at baseline, during the treatment and follow-up. The serum IL-2 level was very low in CHB patients, which was almost undetectable. The levels of Th1-type cytokines including IL-12, TNF-α and IFN-γ were increased while those of Th2-type cytokines including IL-4, IL-6 and IL-10 were decreased at week 48 after treatment (Figure 4). The baseline IP-10 level was increased from week 4 and decreased from week 48 after treatment.

The baseline IL-6 level was correlated with HBV DNA in responders (r = 0.597, P <0.05) but not with HBV DNA in non-responders. IL-10 was correlated with IL-6 (r = 0.762, P = 0.002), and IL-12 was correlated IFN-γ (r = 0.485, P = 0.026).

The IP-10 level was closely correlated with the serum ALT level not only in responders but also in non-responders (r = 0.545, P = 0.005, Figure 5), indicating that IP-10 level fluctuates with serum ALT level. The baseline IP-10 level was lower in patients with their ALT < 40 U/L than in those with their ALT > 40 U/L.

Predictability of IP-10

To determine whether IP-10 can predict the normalization of ALT (< 40 U/L) after peg-IFN α-2b treatment, receiver operating characteristic curve was plotted for IP-10. The

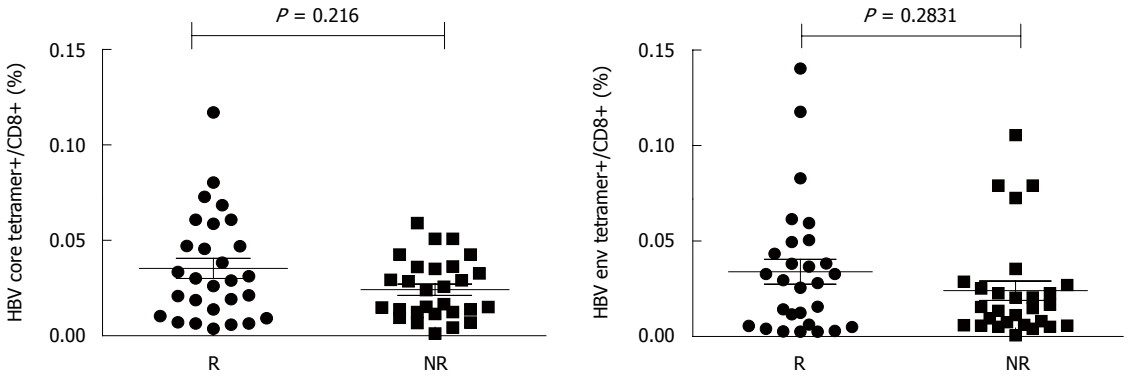

Figure 2 No correlation between increased hepatitis B virus epitope-specific CD8+ T cells and treatment outcome. The frequency of hepatitis B virus (HBV) core or env epitope-specific CD8+ T cells was higher in non-responders (NR) than in responders (R).

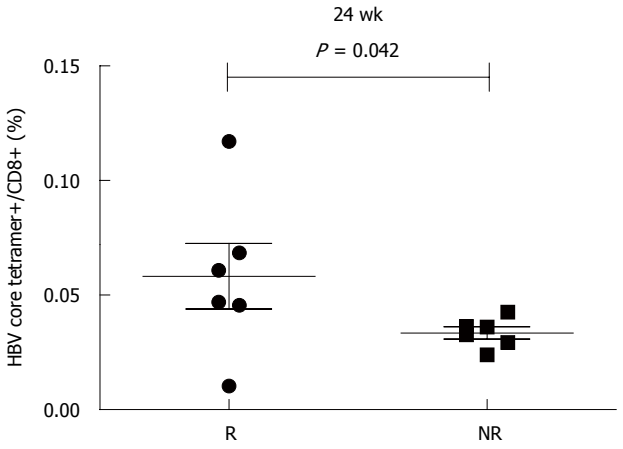

Figure 3 Correlation between increased hepatitis B virus specific T cells and treatment response 24 wk after therapy. The frequency of hepatitis B virus (HBV) core epitope-specific CD8+ T cells at week 24 was higher in responders (R) than in non-responders (NR).

IP-10 level at week 8 after treatment was predictable. The area under the curve was 0.741 ($P = 0.065$). A cutoff value of 437.78 was chosen. Correspondingly, the positive and negative predictive value was 56% and 92%, respectively (Table 2).

DISCUSSION

HBV has a high propensity to persist and several strategies have been developed for control of its evading from T cell responses, including the direct inhibitory effect of viral proteins on T cell responses and the emergence of escape mutations[19-21]. Moreover, HBV infection is more common in immune deficient individuals, such as infants, patients with cancer and those treated with steroid hormone, thereby can interfere with viral clearance by the innate immune system[22,23]. Inefficient innate responses and rapid spread of HBV may in turn delay and impair adaptive responses because of inefficient promotion of T cell priming by innate immunity and through T cell exhaustion induced by a rapidly increased viral load. However, the actual impact of exhaustion by persistent exposure to high antigen concentrations on virus persistence has only been partially defined.

Furthermore, two kinds of drugs (nucleoside analogs and IFN) are usually used in antiviral treatment of CHB

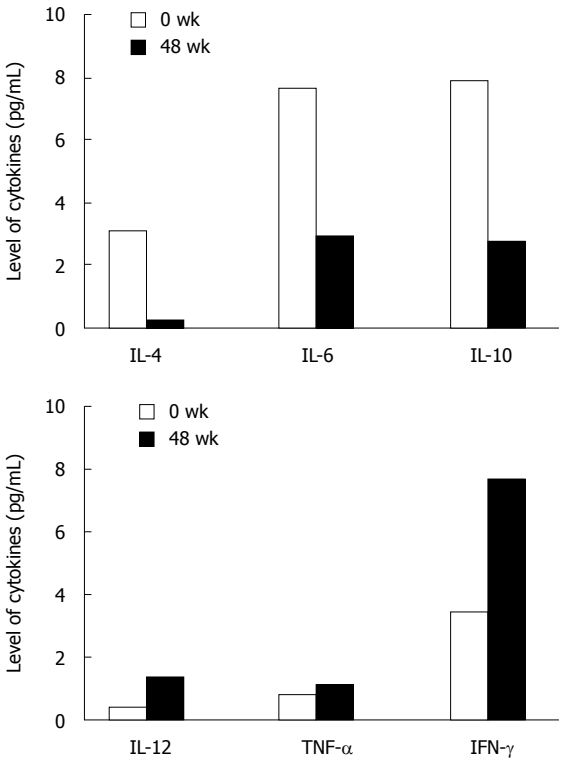

Figure 4 Level of cytokines after treatment. The levels of Th1-type cytokines [interleukin (IL)-12, tumor necrosis factor (TNF)-α and interferon (IFN)-γ] were higher, while the levels of Th2-type cytokines (IL-4, IL-6 and IL-10) were lower at week 48 after treatment.

patients. IFN is involved in numerous immune interactions during viral infection, as an inducer, regulator, and effector of both innate and adaptive antiviral systems. IFN-α and beta are produced rapidly due to viral factors, such as envelope glycoprotein, CpG DNA or dsRNA, and interact with cellular pattern-recognition receptors, such as mannose receptors, toll-like receptors, and cytosolic receptors[24]. In addition, IFN modulates both innate and adaptive immunity, ultimately resulting in an enhanced antiviral effector function.

In the present study, the frequency of HBV epitope-specific CD8+ T cells in peripheral blood was persistently increased at weeks 4, 8, 12 and 24 after peg-IFN α-2a treatment, while the number of HBV epitope-specific CD8 T cells in HBV core 18-27 tetramers and HBV env 335-343

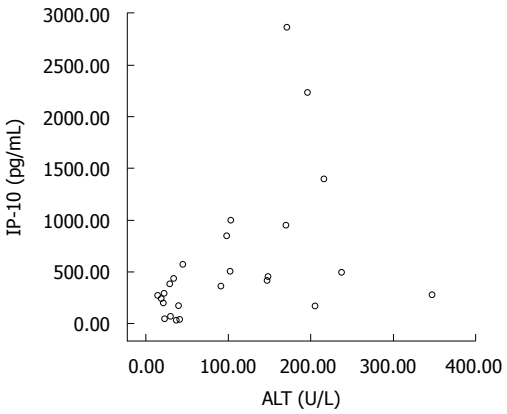

Figure 5 Positive correlation between alanine aminotransferase and inducible protein-10 levels ($r = 0.545$, $P = 0.005$). ALT: Alanine aminotransferase; IP-10: Inducible protein-10.

Table 2 Predictive value of serum inducible protein-10 levels 8 wk after treatment

IP-10 (pg/mL)	ALT < 40 U/L	ALT ≥ 40 U/L	Predictive value
< 437.78	5	4	PPV = 56%
≥ 437.78	1	11	NPV = 92%

IP-10: Inducible protein-10; ALT: Alanine aminotransferase; PPV: Positive predict value; NPV: Negative predict value.

pentamers was greater in responders than in non-responders after pegylated IFN α-2b treatment, suggesting that the therapeutic effect of pegylated IFN α-2b on HBV infection may be attributed to the elevated HBV-specific CD8 T cells, and that the immune response mediated by HBV-specific cells plays an important role in control of HBV. However, the frequency of HBV core 18-27 tetramers+/CD8+ T cells was higher than that of HBV env 335-343 pentamers+/CD8+ T cells after pegylated IFN treatment, suggesting that the HBV core epitope plays a more critical role in induction of a stronger immune response to HBV infection than to HBV env epitope. Pegylated IFN α-2b could enhance specific immune response of CHB patients. Further study should be performed with a large sample size.

Cytokines play an important role in immune modulation. Clearance of HBV infection is mediated by a strong polyclonal cellular response of both CTL and Th1 cells. Chronic HBV infection is caused mainly by an increased response of Th2 cells and impaired production of type 1 cytokines. IL-10, a Th2-type cytokine secreted by T-cells, activated B cells and monocytes, is a powerful inhibitor of Th1 activation and suppresses cell-mediated immunity in mice and humans[25,26]. Of the detected cytokines, Th2-type cytokines such as IL-4, IL-6 and IL-10, were altered conspicuously. After treatment, the level of Th2-type cytokines (IL-4 and IL-10) was down-regulated, thus confirming the immune recover potential of pegylated IFN α-2b, the level of IL-12 which can promote the differentiation of Th1-type cytokines was low, and the production of Th1-type cytokines was increased, indicating that the immune function of pegylated IFN α-2b can be achieved by regulating the balance of Th1/Th2 cytokines.

IL-6 is a multifunctional cytokine with both differentiation and growth-promoting effects for a variety of target cells. IL-6 is generally considered an important cytokine in the network of cytokines that regulate immune reactions and acute phase responses[27]. It was reported that IL-6 is correlated with liver fibrosis/cirrhosis[28] and is a cell attachment site for HBV[29]. In the present study, the IL-6 level was correlated with HBV DNA plasma only in responders.

IP-10, a chemotactic CXC chemokine of 77 aa in its mature form[30,31], can be produced by a variety of cells, including hepatocytes[32,33]. The correlation between IP-10 levels and necroinflammatory activity, as well as the high and low IP-10 levels before and after pegylated IFN α-2b treatment, may imply that IP-10 plays a role in the natural pathogenesis of HBV-induced liver damage[34]. It was reported that the baseline IP-10 level can predictive the response of CHB patients to HCV treatment, and is correlated with liver inflammation and fibrosis[35,36]. In this study, the baseline IP-10 level in CHB patients could predict the normalization of ALT after pegylated IFN α-2b treatment.

In conclusion, given the importance of protective T cell responses in control of HBV, the correlation between immunomodulatory molecules and pegylated IFN α-2b treatment in restoration of the immune responses of antiviral T cells are highly desirable. Pegylated IFN α-2b therapy can enhance the immune response of CHB patients by influencing the production of cytokines. IP-10 can potentially predict the normalization of ALT, which is correlated with liver damage. Further study is needed with a large sample size.

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COMMENTS

Background

More than two billion people have been infected with hepatitis B virus (HBV) and chronic HBV infection affects about 400 million people worldwide. Two kinds of drugs [nucleoside analogs and interferon (IFN)] are mainly used in treatment of chronic hepatitis B (CHB) patients. IFN is involved in numerous immune interactions as an inducer, regulator, and effector in treatment of viral infections. Cytokines play an important role in immune modulation. Clearance of HBV infection is mediated by a strong polyclonal cellular response of both CTL and Th1 cells. Chronic HBV infection is caused mainly by an increased response of Th2 cells and impaired production of type 1 cytokines. Inducible protein 10 (IP-10) is a chemotactic CXC chemokine of 77 aa in its mature form.

Research frontiers

IFN-α and β are produced rapidly due to viral factors, such as envelope glycoproteins, CpG DNA or dsRNA, and interact with cellular pattern-recognition receptors, such as mannose receptors, toll-like receptors, and cytosolic receptors. IP-10 can be produced by a variety of cells, including hepatocytes. The results of this study show that the baseline IP-10 level can predict the response of patients with HBV infection to its treatment with pegylated IFN α-2b.

Innovations and breakthroughs

The present study demonstrated the correlation between pegylated IFN α-2b treatment and HBV-specific T lymphocytes. In addition, the effect of pegylated IFN α-2b on HBV infection could be achieved by balancing the production of Th1/Th2 cytokines and IP-10 could predict the outcome of patients with HBV infection after pegylated IFN α-2b treatment.

Applications

In this study, pegylated IFN α-2b could up-regulate HBV epitope specific CD8+ T cells. The specific cellular immune response could control HBV. IP-10 serum

level could predict the outcome of patients with HBV infection after pegylated IFN α-2b treatment, thus providing a new index for the treatment of HBV infection. Pegylated IFN α-2b may be used as a novel strategy for the treatment of HBV infection by regulating the cytokines.

Terminology

Human leukocyte antigen (HLA) typing is a method to define the HLA+ and HLA+ blood for studied subjects. Flow cytometry is used to define the HBV epitope specific CD8+ T lymphocytes. Cytometric bead assay is a new technique for detecting serum concentration of cytokines.

Peer review

This is a very interesting study, showing that pegylated IFN α-2b therapy can increase the frequency of specific CD8+ T lymphocytes in CHB patients. This may contribute to the better control of HBV replication and to the recovery of CHB patients, thus having a promise for therapeutic interventions. The experiments support the claim of the authors.

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