Transforming Growth Factor - β Level Might Be An Independent Factor Related to Occurrence of Chronic Hepatitis B

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Abstract

To investigate association between immune cell-related cytokines and development of chronic hepatitis B (CHB). Patients with chronic hepatitis B virus (HBV) infection in immune tolerance (IT, n=30) and hepatitis B envelope antigen (HBeAg) positive CHB (n=250) were enrolled in the study. HBV virus, serological indicators, and plasma cytokine levels were detected at the time of enrollment. The results showed that there were significant differences in median age of patients (27 vs. 31y), alanine aminotransferase level (ALT, 29.85 vs 234.70 U/L), alanine aminotransferase level (AST, 23.40 vs. 114.90 U/L), HBsAg level (4.79 vs. 3.88 log₁₀ IU/ml), HBeAg (1606.36 vs. 862.47 S/CO) and HBV DNA load (8.17 vs 6.71 log₁₀ IU/ml) between IT and CHB groups (all $P < 0.01$). The median values of Fms-like tyrosine kinase 3 ligand (FLT3-L), interferon- γ (IFN- γ), interleukin- 17A (IL-17A) and transforming growth factor- beta (TGF- β 1) in IT group were significantly higher than those in CHB group (FLT3-L: 41.62 vs. 27.47 pg/ml; IFN- γ : 42.48 vs. 33.18 pg/ml; IL-17A: 15.66 vs. 8.90 pg/ml; TGF- β 1: 4921.50 vs. 2234 pg/ml. All $P < 0.01$). The median values of IFN- α 2, TGF- β 3 and IL-10 levels in IT group were significantly lower than those in CHB group (IFN- α 2: 15.24 vs. 35.78 pg/ml, $P = 0.000$; TGF- β 3: 131.69 vs. 162.61 pg/ml, $P = 0.025$; IL-10: 5.02 vs. 7.9 pg/ml, $P = 0.012$). The multivariate logistic regression analysis indicated that TGF- β 1 (OR=0.999, 95% CI 0.999-1.000, $P < 0.001$) and TGF- β 2 levels (OR=1.008, 95%CI 1.004-1.012, $P < 0.001$) were significantly associated with the incidence of CHB. The results suggest that TGF- β level might be an independent factor related to the occurrence of CHB.

Introduction

There have been increasing in hepatitis B virus (HBV) related morbidity and mortality over the past decades, representing the 7th most frequent cause of death worldwide^[1]. Chronic hepatitis B (CHB) is an immune-mediated disease caused by the immune reaction initiated by HBV, which causes necrosis of hepatocytes and inflammation of liver tissue, damage of liver tissue while eliminating the virus. If there were no immune clearance, long-term HBV infection would not cause damage of liver tissue^[2,3]. Therefore, the occurrence of CHB is the result of immune interaction between the virus and its stimulation.

Chronic HBV infection is characterized by quantitative and functional defects of virus-specific T-cell response which is essential for a persistent control of infection. Recovery from HBV infection is dependent on the presence of dendritic cells (DCs), natural killer (NK) cells, CD4⁺ T lymphocytes cells, and CD8⁺ T lymphocytes cells, combined with the cytokines secreted by them. The abnormalities of helper T lymphocytes (TH), DC and cytokines in the cellular immunity of patients with CHB are closely related to the pathogenesis and chronicity of hepatitis B^[4-6].

The immune tolerance of patients with chronic HBV infection and the difficulty in recovery from infection in patients with CHB are related to the deficiency of HBV-specific T cell functions. However, the role of many other immune cells and their cytokines in pathogenesis of CHB is not clear. We detected cytokines that cause liver inflammation and immunosuppression (IL-6, IL-10 and TGF- β), factors that stimulate

immune cell function (IFN- α), factors that associate with virus removal (IL-17A and IFN- γ) or stimulate proliferation of DC cells and NK cells (Flt3-L).^[7-9] The purpose of this study was to investigate the correlation between immune cell-related cytokines and development of chronic HBV infection.

Materials And Methods

Ethic statement

This is a prospective study in immune tolerant patients (IT) with chronic HBV infection and hepatitis B envelope antigen (HBeAg) positive CHB. To minimize selection bias, we enrolled all eligible patients who attended the Department of Hepatology, Beijing Ditan Hospital during August 2015 and May 2017. The study was approved by the Ethics Committee of Beijing Ditan Hospital (JDL-2017-034-01) according to the guidelines of the Declaration of Helsinki and registered at clinicaltrials.gov (NCT03210506). All participants provided written informed consent.

Inclusion and exclusion criteria

None of the subjects received treatment prior to the clinical tests involved in this study.

The criteria of immunological tolerance (IT) with chronic HBV infection were: 1) persistent HBsAg positive (HBsAg $\geq$ 0.05 IU/ml) > 6 months; 2) high HBeAg ($\geq$ 1200 S/CO) level; 3) high HBV DNA load ($\geq 10^7$ IU/ml); 4) persistent normal ALT (ALT < 40 U/L), or mild inflammation and fibrosis in histological examination; 5) age 18-40 years; 6) no hormone and/or immunosuppressive agents and other liver protection medicines;

The criteria of HBeAg positive CHB were: 1) persistent HBsAg positive (HBsAg $\geq$ 0.05 IU/ml) > 6 months; 2) HBeAg positive, HBeAg $\geq$ 1.0 S/CO; 3) HBV DNA positive ($\geq 10^4$ IU/ml); 4) ALT abnormal ($\geq$ 80 U/L) for more than 3 months, or obvious inflammation in liver by histological examination; 5) age 18-65 years; 6) no hormones and/or immunosuppressants and other hepatoprotective drugs;

Exclusion criteria included: 1) co-infection with other hepatitis viruses such as hepatitis C virus and hepatitis D virus; 2) autoimmune liver disease; 3) co-infection with other viruses such as Epstein-Barr virus, cytomegalovirus, HIV infection; 4) chronic alcohol abuse and/or other liver-damaging drugs; 5) mental illness; 6) evidence of liver neoplasms: liver cancer or Alpha-fetoprotein (AFP) > 100 ng/ml; 7) exclusion of hepatic fibrosis and cirrhosis by Fibroscan test;^[10] 8) absence of long-term follow up for serious diseases of other systems such as heart, brain, lung, and kidney; 9) presence of hormones and/or immunosuppressants and other protective measures; 10) presence of other liver diseases (such as fatty liver, metabolic liver disease, and liver tumors).

HBV DNA load and HBV serological markers

Serum HBV DNA load was determined using Roche Cobas AmpliPrep/Cobas TaqMan 96 full automatic real-time fluorescence quantitative PCR detection reagent with a lower limit of 20 IU/ml (Roche,

Pleasanton, CA, USA). HBV markers were measured by Abbott Architect i2000 detection reagent (Abbott Diagnostics, Abbott Park, IL, USA). Liver and kidney functions were assessed with a Hitachi 7600 automatic biochemical analyzer (Hitachi 7600-020, Japan).

Quantitative detection of plasma cytokines

The levels of Fms-like tyrosine kinase 3 ligand (FLT3-L), interferon alpha 2 (IFN- α 2) and IFN- γ , interleukin-10 (IL-10), IL-17A, IL-6, TNF- α , transforming growth factor- β 1 (TGF- β 1), TGF- β 2, and TGF- β 3 were determined at the time of enrollment by Luminex technique.

Statistical analysis

Normal distribution data was expressed as mean $\pm$ SD. Comparison between two groups was made by variance analysis and independent sample *t*-test, and the comparison of the data within the group was completed by two independent sample *t*-test. Non-normal distribution data were expressed by median and Q1Q3. Mann-Whitney *U* test or Wilcoxon signed rank test was used for inter-group or intra-group comparison. Multiple logistic regression analysis was used to analyze the correlation between detection indexes and hepatitis after HBV infection. The data was analyzed by SPSS (Chicago, IL) and Graphpad Prism 5 software.

Results

Patient baseline clinical characteristics

A total of 280 patients (male 163, 67.2%; female 117, 32.8%; median age 31 y), including 30 chronic HBV infection in immune tolerance (IT group, *n*=30) and hepatitis B envelope antigen (HBeAg) positive CHB (CHB group, *n*=250), were enrolled. The basic demographic and clinical characteristics of the subjects were shown in Table 1 and Figure 1. There were significant differences in the median age (27 vs. 31y, *P*=0.004; *Z*= 3.893), ALT level (29.85 vs. 234.70 U/L, *P*=0.000; *Z* = 8.488), AST level (23.40 vs. 114.90 U/L, *P*=0.000; *Z*=8.493), HBsAg level (4.79 vs. 3.88 log₁₀ IU/ml, *P*=0.000; *Z* = -7.820), HBeAg (1606.36 vs. 862.47 S/CO, *P*= 0.000; *Z*=-8.108) and HBV DNA load (8.17 vs 6.71 log₁₀ IU/ml, *P*=0.000; *Z*=6.762) between the IT and CHB group. In the CHB group, 143 patients had a worsening ALT level (5 times higher than the upper limit of normal), 57 patients had flare (3-5 times the upper limit of normal), the remained 50 patients had obvious chronic liver inflammation (1-3 times the upper limit of normal value).

Cytokine levels in IT and CHB

The levels of Flt3-L, IFN- α 2, IFN- γ , IL-10, IL-17A, IL-6, TGF- β 1, TGF- β 2 and TGF- β 3 were shown in Table2 and Figure2. The median levels of Flt3-L, IFN- γ , IL-17A, and TGF- β 1 in IT group were significantly higher than those in CHB group (Flt3-L: 41.62 vs. 27.47 pg/ml, *P*=0.008/*Z*=-2.536; IFN- γ : 42.48 vs. 33.18 pg/ml, *P*=0.008/*Z*=-2.636; IL-17A: 15.66 vs. 8.90 pg/ml, *P*=0.002/*Z*=-3.037; TGF- β 1: 4921.50 vs. 2234 pg/ml, *P*=0.000, *Z*=-4.404), while the median levels of IFN- α 2, TGF- β 3, and IL-10 in the IT group were significantly

lower than those in the CHB group (IFN- α 2: 15.24 vs 35.78 Pg/ml, $P = 0.000/Z = 3.727$; TGF- β 3: 131.69 vs 1.62.61 pg/ml, $P=0.025$, $Z=2.245$; IL-10: 5.02 vs. 7.9 pg/ml, $P=0.012/Z=2.498$).

we divided CHB patients into three groups as follows :Group A, 50 patients had obvious chronic liver inflammation (1-3 times the upper limit of normal value) Group B, 57 patients had flare (3-5 times the upper limit of normal) Group C, 143 patients had a worsening ALT level (5 times higher than the upper limit of normal). The cytokine levels among the three groups were shown in Table s1. The median levels of IL-10 in group A were significantly lower than those in group B and C (respectively 5.070 vs 10.920 Pg/ml, $P=0.021/Z = -2.304$; 5.070 vs 9.170 Pg/ml, $P = 0.003/Z = -2.954$). The median levels of IFN- γ in group A were significantly lower than those in group B (12.940 vs 42.000 Pg/ml, $P=0.028/Z = -2.203$).

3. Logistic regression analysis of risk factors of chronic HBV infection

We included all cytokines in the risk factor analysis and compared with immune tolerant patients. All cytokines were not statistically significant in univariate logistic regression analysis, but in adjusted multivariate logistic regression analysis, TGF- β 1 and TGF- β 2 were found to be significant (OR= 0.999, 95% CI 0.999-1.000, $P < 0.001$; OR=1.008, 95% CI 1.004-1.012, $P < 0.001$, Table 3).

Table 1. Clinical characteristics between the two groups

	All(n=280)	IT(n=30)	CHB(n=250)	P (IT vs CHB)
Male(%)	163(67.2%)	14/16(87.5%)	149/101(59.6%)	$P=0.175$ $\chi^2=1.842$
Age(y)	31(28, 37.75)	27(25.75, 31.25)	31(28, 37.25)	$P=0.004$ $Z=3.893$
ALT(U/L)	234.70(129.30, 355.28)	29.85(21.83, 39.85)	234.70(129.30, 354.75)	$P=0.000$ $Z=8.488$
AST(U/L)	113.80(63.40, 174.40)	23.40(19.45, 26.83)	114.90(63.40, 174.40)	$P=0.000$ $Z=8.493$
TBIL(μ mol/L)	14.30(11.63, 20.10)	NA	14.30(11.63, 20.10)	NA
ALB(g/L)	45.20(42.60, 47.40)	NA	45.20(42.60, 47.40)	NA
HBsAg(log10 IU/ml)	3.88(3.61, 4.11)	4.79(4.59, 4.93)	3.88(3.62, 4.12)	$P=0.000$ $Z=-7.820$
HBeAg(S/CO)	861.26(468.04, 1195.97)	1606.36(1556.53, 1679.44)	862.47(469.18, 1196.43)	$P=0.000$ $Z=-8.108$
HBV DNA(log10 IU/ml)	6.71(6.34, 7.39)	8.17(7.75, 8.42)	6.71(6.35, 7.39)	$P=0.000$ $Z=6.762$

Table 2. The comparison of Cytokine levels between IT and CHB groups

	All(n=280)	IT(n=30)	CHB(n=250)	<i>P</i> (IT vs CHB)
FIT3-L (pg/ml)	29.26(13.42, 83.99)	41.62(21.47, 124.87)	27.47(12.56, 82.42)	<i>P</i> =0.008; <i>Z</i> =-2.536
IFN-a2 (pg/ml)	32.35(9.12, 67.54)	15.24(4.07, 30.73)	35.78(10.59, 68.80)	<i>P</i> =0.000; <i>Z</i> =3.727
IFN-γ (pg/ml)	33.99(12.94, 93.03)	42.48(26.24, 111.56)	33.18(5.02, 93.03)	<i>P</i> =0.008; <i>Z</i> =-2.636
IL-10 (pg/ml)	7.26(3.08, 17.00)	5.02(2.98, 10.13)	7.92 (3.25, 18.03)	<i>P</i> =0.012; <i>Z</i> =2.498
IL-17A (pg/ml)	12.30(3.62, 44.74)	15.66 (9.84, 44.46)	8.90(3.34, 45.06)	<i>P</i> =0.002; <i>Z</i> =-3.037
IL-6 (pg/ml)	2.28(1.10, 9.32)	3.17 (1.53, 18.33)	2.26(0.98, 9.29)	<i>P</i> =0.061; <i>Z</i> =-1.872
TGFβ1 (pg/ml)	2274.50(3.63, 4751.75)	4921.50 (3840.75, 7689.50)	2234 (3.56, 3387)	<i>P</i> =0.000; <i>Z</i> =-4.404
TGFβ2 (pg/ml)	425.81(348.70, 647.41)	419.01 (311.80, 580.51)	435.48 (348.70, 681.08)	<i>P</i> =0.398; <i>Z</i> =0.846
TGFβ3 (pg/ml)	156.35(131.69, 198.01)	131.69 (112.78, 171.94)	162.61 (135.16, 207.50)	<i>P</i> =0.025; <i>Z</i> =2.245

Table 3. Logistic regression analysis of risk factors of chronic HBV infection

Single factor logistic regression analysis		
cytokines	OR(95%CI)	<i>P</i> value
Flt3-L	1.000(0.9981-0.001)	0.715
IFN- α_2	1.008(0.999-1.017)	0.083
IFN- γ	0.999(0.998-1.000)	0.188
IL-10	1.045(1.000-1.092)	0.052
IL-17A	0.999(0.996-1.003)	0.774
IL-6	1.002(0.995-1.008)	0.664
TGF- β 1	1.000	0.088
TGF- β 2	1.000(0.999-1.001)	0.889
TGF- β 3	1.008(0.999-1.018)	0.097
Multiple factor Logistic regression analysis		
TGF- β 1	0.999(0.999-1.000)	<i>P</i> < 0.001
TGF- β 2	1.008(1.004-1.012)	<i>P</i> < 0.001

OR: Odds ratio; 95% CI: confidence interval

Discussion

In this study, liver function parameters, HBV DNA load, HBeAg and HBsAg levels were compared in patients with chronic HBV infection in immune tolerant phase and HBeAg chronic hepatitis B. The results showed that there were significant differences in age, HBV DNA load, HBsAg level, HBeAg level and ALT level between the two groups. The levels of HBV DNA, HBsAg and HBeAg in patients with chronic hepatitis were significantly lower than those in patients with immune tolerance, while ALT was significantly higher than those in patients with tolerance. It may be related to the immune reaction of hepatitis. The main manifestations of liver inflammation are necrosis of hepatocytes and inflammation of liver tissue. Liver necrosis in hepatitis leads to the reduction of HBV replication and the production of viral antigen, so the level of HBV DNA, HBeAg and HBsAg are decreased clinically. In general, the higher the level of ALT, the more hepatocyte necrosis and liver tissue inflammation, resulting in a bigger decline in the HBV DNA load, HBeAg and HBsAg levels^[11,12].

Immunotolerant patients showed high HBV DNA load, high HBeAg and high HBsAg levels and normal ALT levels, of which high HBV DNA load and high HBsAg levels were crucial. Stable and high levels of serum HBsAg (~ 5 log 10 IU/ML) and HBV DNA (> 8 log 10 IU/ML) are the hallmark features of Asian immune tolerance. ^[13] The positive predictive value of HBsAg levels > 25000 IU/ml in predicting liver fibrosis < F1

is greater than 90%.^[14] Our previous studies showed that the cutoff value of HBsAg level in patients diagnosed with immune tolerance was 4.31 log₁₀ IU/ml.^[15] In this study, HBV DNA load in patients with immune tolerance was 8.17 log₁₀ IU/ml (7.75 ~ 8.42), HBsAg level was 4.79 log₁₀ IU/ml (4.59 ~ 4.93), and ALT level was 29.85(21.83-39.85)U/L, all of which were consistent with previous studies. High HBV DNA load, high HBeAg and HBsAg levels in patients with immune tolerance may also be related to the lack of immune response,^[16] that is, there is a specific T cell response to HBV, but not enough to achieve viral clearance.^[17] In HBeAg-positive CHB patients, HBsAg and HBeAg can inhibit the secretion of surface functional molecules and cytokines in a variety of immune cells, which plays a role in inducing immune tolerance to HBV infection.^[18]

In this study, although the levels of Flt3-L, IFN-γ and IL-17A in patients with chronic hepatitis B were significantly lower than those in patients with immune tolerance, the levels of IFN-α and IL-10 in patients with chronic hepatitis B were significantly higher than those in patients with immune tolerance. The increase of IFN-α may be the reason of breaking immune tolerance and hepatitis in IT patients.^[19,20] The increase of IL-10 level and TGF-β in patients with CHB can inhibit the immune response and impede the spontaneous recover from chronic hepatitis B. The significant decrease in level of FLT3-L, IFN-γ, and IL-17A could lead to the prolonged and continuous progress of CHB, which indicates the time for antiviral therapy. Our study also showed that CHB patients with low ALT level had significantly lower levels of IL-10 and IFN-γ than those with high ALT level. CHB patients with high ALT have higher NK cell activity, and NK cells can secrete IFN-γ, so CHB patients with high ALT have higher IFN-γ.^[21] The liver inflammation of CHB patients with high ALT was obvious. Mononuclear macrophages are important immune cells involved in liver inflammation, and the increased activity of these mononuclear macrophages leads to an increase in IL-10 level.^[9,22]

All cytokines were included in the analysis of risk factors for CHB patients with chronic HBV infection in immune tolerant phase and chronic hepatitis B. In multivariate logistic regression analysis, TGF-β 1 and TGF-β 2 were statistically significant ($P < 0.001$). Only elevated levels of TGF-β alone lead to persistent liver inflammation and the development of liver fibrosis and cirrhosis. It is hard to explain the cause of chronic hepatitis B by using the detected cytokines alone. What's more, given our limited sample size, the conclusion need be verified in a large cohort in future.

In conclusion, the results suggest that TGF-β level might be an independent factor associated with the development of chronic hepatitis B. The finding provide a clue for identifying the optimal timing of treatment for chronic HBV infection in clinical practice. The relatively small sample size of this study may cause some deviation to the results. In the future, we will expand the sample size to correct this deviation.

Declarations

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Compliance with ethical standards

Conflict of interest All authors declare that they have no competing interest.

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Figures

Figure 1

Comparison of clinical characteristics between IT and CHB groups.

Figure 2

The comparison of Cytokine levels between IT and CHB groups Note: A. Flt3-L level between the two groups; B. IFN- α 2 level between the two groups; C. IFN- γ level between the two groups; D. IL-10 level between the two groups; E. IL-17A level between the two groups; F. IL-6 level between the two groups; G. TGF- β 1 level between the two groups; H. TGF- β 2 level between the two groups. ns: no significance.

Supplementary Files

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