Transforming growth factor β as a possible independent factor in chronic hepatitis B

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Abstract
To investigate the association between immune-cell-related cytokines and the development of chronic hepatitis B (CHB), patients with chronic hepatitis B virus (HBV) infection in the immunotolerant (IT) phase (n = 30) or hepatitis B envelope antigen (HBeAg)-positive CHB (n = 250) were enrolled in this study. Serological indicators and plasma cytokine levels were measured at the time of enrollment. The results showed that there were significant differences in the median age of the patients (27 vs. 31 years), alanine aminotransferase levels (ALT, 29.85 vs. 234.70 U/L), alanine aminotransferase levels (AST, 23.40 vs. 114.90 U/L), HBsAg levels (4.79 vs. 3.88 log₁₀ IU/ml), HBeAg levels (1606.36 vs. 862.47 S/CO), and the HBV DNA load (8.17 vs. 6.71 log₁₀ IU/ml) between the IT and CHB groups (all \( P < 0.01 \)). The median values of Fms-like tyrosine kinase 3 ligand (FLT3-L), interferon-gamma (IFN-γ), interleukin-17A (IL-17A), and transforming growth factor beta (TGF-β1) were significantly higher in the IT group than in the 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-β1, 4921.50 vs. 2234 pg/ml; all \( P < 0.01 \)). The median IFN-α2, TGF-β3 and IL-10 levels in the IT group were significantly lower than those in the CHB group (IFN-α2, 15.24 vs. 35.78 pg/ml, \( P = 0.000 \); TGF-β3, 131.69 vs. 162.61 pg/ml, \( P = 0.025 \); IL-10, 5.02 vs. 7.9 pg/ml, \( P = 0.012 \)). Multivariate logistic regression analysis indicated that TGF-β 1 (OR = 0.999, 95% CI 0.999-1.000, \( P < 0.001 \)) and TGF-β2 levels (OR = 1.008, 95%CI 1.004-1.012, \( P < 0.001 \)) were modestly but 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
The rate of hepatitis B virus (HBV)-related morbidity and mortality has increased over the past decades and is now the seventh most frequent cause of death worldwide [1]. Chronic hepatitis B (CHB) is an immune-mediated disease caused by the immune reaction to HBV, which causes necrosis of hepatocytes, inflammation of liver tissue, and liver damage while the virus is being eliminated. If there were no immune clearance, long-term HBV infection would not cause damage to liver tissue [2, 3]. Therefore, the CHB is the result of the interaction between the virus and the immune system.

Chronic HBV infection is characterized by quantitative and functional defects in the virus-specific T-cell response, which is essential for control of persistent infection. Recovery from HBV infection is dependent on the presence of dendritic cells (DCs), natural killer (NK) cells, CD4⁺ T lymphocytes, and CD8⁺ T lymphocytes, in combination with the cytokines secreted by these cells. Abnormalities in helper T lymphocytes (Th), DCs, and cytokines in the cellular immunity of patients with CHB are closely associated with the pathogenesis and chronicity of hepatitis B [4–6].

Immune tolerance in patients with chronic HBV infection and difficulty in recovering from infection in patients with CHB are related to deficiencies in HBV-specific T cell functions. However, the role of many other immune cells and their cytokines in the pathogenesis of CHB is not clear.
We measured the levels of cytokines that cause liver inflammation and immunosuppression (IL-6, IL-10 and TGF-β), factors that stimulate immune cell function (IFN-α), factors that are associated with elimination of the virus (IL-17A and IFN-γ), and factors that stimulate the proliferation of DCs and NK cells (Flt3-L) [7–9]. The purpose of this study was to investigate the correlation between immune-cell-related cytokines and the development of chronic HBV infection.

Materials and methods

Ethics statement

This is a prospective study in immunotolerant (IT) patients with chronic HBV infection and hepatitis B envelope antigen (HBeAg)-positive CHB patients. To minimize selection bias, we enrolled all eligible patients who attended the Department of Hepatology of Beijing Ditan Hospital between 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 for immunological tolerance (IT) with chronic HBV infection were: (1) persistent HBsAg positivity (HBsAg ≥ 0.05 IU/ml) > 6 months, (2) a high HBeAg level (≥ 1200 S/CO), (3) a high HBV DNA load (≥ 10^7 IU/ml), (4) a consistently normal ALT level (ALT <40 U/L) or mild inflammation and fibrosis in the histological examination, (5) age 18–40 years, and (6) no treatment with hormones, immunosuppressive agents, or hepatoprotective drugs.

The criteria for HBeAg-positive CHB were (1) persistent HBsAg positivity (HBsAg ≥ 0.05 IU/ml) for longer than 6 months, (2) HBeAg positivity (HBeAg ≥ 1.0 S/CO), (3) HBV DNA positivity (≥ 10^4 IU/ml), (4) abnormal ALT (≥ 80 U/L) for more than 3 months or obvious inflammation of the liver in the histological examination, (5) age 18–65 years, (6) no treatment with hormones, immunosuppressants, or hepatoprotective drugs.

Exclusion criteria included (1) coinfection with other hepatitis viruses such as hepatitis C virus or hepatitis D virus, (2) autoimmune liver disease, (3) coinfection with other viruses such as Epstein-Barr virus, cytomegalovirus, or human immunodeficiency virus (HIV), (4) chronic alcohol abuse and/or the use of liver-damaging drugs, (5) mental illness, (6) evidence of liver neoplasms or liver cancer or alpha-fetoprotein (AFP) levels above 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 or other protective measures, (10) presence of other liver diseases (such as fatty liver, metabolic liver disease, or liver tumors).

HBV DNA load and HBV serological markers

The 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 using Abbott Architect i2000 detection reagent (Abbott Diagnostics, Abbott Park, IL, USA). Liver and kidney functions were assessed using 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-α2), IFN-γ, interleukin 10 (IL-10), IL-17A, IL-6, TNF-α, transforming growth factor-β1 (TGF-β1), TGF-β2, and TGF-β3 were determined at the time of enrollment by the Luminex technique.

Statistical analysis

Normal distribution data are expressed as the mean ± SD. Comparisons between two groups were made by variance analysis and independent-samples t-test, and comparisons of the data within a group were made using the independent-samples t-test. Non-normal distribution data were expressed as the median and Q1Q3. The Mann Whitney U test or the Wilcoxon signed rank test was used for inter-group or intra-group comparisons. Multiple logistic regression analysis was used to analyze the correlation between detection rates and hepatitis after HBV infection. The data were analyzed using SPSS (Chicago, IL) and GraphPad Prism 5 software.

Results

Baseline clinical characteristics of patients

A total of 280 patients (163 male, 67.2%; 117 female, 32.8%; median age, 31 y), including 30 with chronic HBV infection in the immunotolerance phase (IT group, n = 30) and 250 with HBeAg-positive CHB (CHB group, n = 250) were enrolled. The basic demographic and clinical characteristics of the subjects are shown in Table 1 and Figure 1. There were significant differences in the median age (27 vs. 31 y,
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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 level (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 groups. In the CHB group, 143 patients had a worsening ALT level (5 times higher than the upper limit of normal), 57 patients were experiencing a flare (3-5 times the upper limit of normal), and the remaining 50 patients had obvious chronic liver inflammation (1-3 times the upper limit of normal).

Cytokine levels in IT and CHB

The levels of Flt3-L, IFN-α2, IFN-γ, IL-10, IL-17A, IL-6, TGF-β1, TGF-β2, and TGF-β3 are shown in Table 2 and Figure 2. The median levels of Flt3-L, IFN-γ, IL-17A, and TGF-β1 in the IT group were significantly higher than those in the 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-10, 7.114 vs. 2.107 pg/ml, P = 0.000/Z = -4.404), while the median levels of IFN-α2, TGF-β3, and IL-10 in the IT group were significantly lower than those in the CHB group (IFN-α2, 15.24 vs. 35.78 pg/ml, P = 0.000/Z = 3.727; TGF-β3, 131.69 vs. 162.61 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 the CHB patients into three groups as follows: group A included 50 patients with obvious chronic liver inflammation (1-3 times the upper limit of normal value), group B included 57 patients experiencing a flare (3-5 times the upper limit of normal), and group C included 143 patients with worsening ALT level (5 times higher than the upper limit of normal). The cytokine levels among the three groups are shown in Supplementary Table S1. The median levels of IL-10 in group A were significantly lower than those in groups B and C (5.070 vs. 10.920 pg/ml, P = 0.021/Z = -2.304 and 5.070 vs. 9.170 pg/ml, P = 0.003/Z = -2.954, respectively). 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).

Logistic regression analysis of risk factors for chronic HBV infection

We included all cytokines in the risk factor analysis and compared their levels with those in immunotolerant patients. No statistically significant differences were found in the levels of any of the cytokines in univariate logistic regression analysis, but in adjusted multivariate logistic regression analysis, differences in TGF-β 1 and TGF-β 2 levels 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).

Discussion

In this study, liver function parameters, the HBV DNA load, and HBeAg and HBsAg levels in patients with chronic HBV infection in the immunotolerant phase and in those with HBeAg-positive chronic hepatitis B were compared. The results showed that there were significant differences in age, HBV DNA load, HBeAg level, HBeAg level, and ALT level between the two groups. The levels of HBV DNA, HBeAg, and HBeAg in patients with chronic hepatitis were significantly lower than those in patients with immune tolerance, while ALT was significantly higher than in patients with tolerance. This may be related to the immune reaction to infection. The main manifestations of liver inflammation are necrosis of hepatocytes and inflammation of liver tissue. Liver necrosis in hepatitis leads to a reduction in HBV replication and the production of viral antigens, so the clinical levels of HBV DNA, HBeAg, and HBsAg decrease. In general, the higher the level of ALT, the more hepatocyte necrosis and liver tissue inflammation is occurring, resulting

| Table 1 Clinical characteristics of the two groups

|            | All (n = 280) | IT (n = 30) | CHB (n = 250) | (IT vs. CHB) |
|------------|--------------|------------|---------------|--------------|
| Male (%)   | 163 (67.2%)  | 14/16 (87.5%) | 149/101 (59.6%) | P = 0.175; Z = 1.842 |
| Age (years)| 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, 17440) | 23.40 (19.45, 26.83) | 114.90 (63.40, 174.40) | P = 0.000; Z = 8.493 |
| TBIL (µmol/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 |
in a larger decline in the HBV DNA load and HBeAg and HBsAg levels [6, 11].

Immunotolerant patients had a high HBV DNA load, high HBeAg levels, high HBsAg levels, and normal ALT levels. Of these, the high HBV DNA load and high HBsAg levels are 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 immune tolerance in Asian patients [12]. The positive predictive value of HBsAg levels > 25,000 IU/ml in predicting liver fibrosis < F1 is greater than 90% [13]. Our previous studies showed that the cutoff value for HBsAg in patients diagnosed with immune tolerance was 4.31 log_{10} IU/ml [14]. In this study, the mean HBV DNA load in patients with immune tolerance was 8.17 log_{10} IU/ml (7.75 - 8.42), the HBsAg level was 4.79 log_{10} IU/ml (4.59 - 4.93), and the ALT level was 29.85 (21.83-39.85) U/L, all of which are consistent with previous studies. A high HBV DNA load and high HBeAg and HBsAg levels in patients with immune tolerance may also be related to the lack of an immune response [15], that is, there is a specific T-cell response to HBV infection, but not enough to achieve viral clearance [16]. In HBeAg-positive CHB patients, HBsAg and HBeAg can inhibit the secretion of functional surface molecules and cytokines in a variety of immune cells, which plays a role in inducing immune tolerance to HBV infection [17].

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 in IFN-α may be the reason for breaking immune tolerance and hepatitis in IT patients [18, 19]. An increase in IL-10 and TGF-β levels in patients with CHB can inhibit the immune response and impede spontaneous recovery from chronic hepatitis B. A significant decrease in the levels of FLT3-L, IFN-γ, and IL-17A could lead to a prolonged and continuous progress of CHB, which determines the time required for antiviral therapy. Our study also showed that CHB patients with low ALT levels had

![Graphs and tables showing comparisons between IT and CHB groups](https://via.placeholder.com/150)

**Fig. 1** Comparison of clinical characteristics between the IT and CHB groups

**Table 2** Comparison of cytokine levels between the IT and CHB groups

|                | All (n = 280) | IT (n = 30)      | CHB (n = 250) | P (IT vs. CHB) |
|----------------|--------------|-----------------|--------------|----------------|
| Flt3-L (pg/ml) | 29.26 (13.42, 83.99) | 41.62 (21.47, 124.87) | 27.47 (12.56, 82.42) | P = 0.008; Z = -2.536 |
| IFN-α2 (pg/ml) | 32.35 (9.12, 67.54) | 15.24 (4.07, 30.73) | 35.78 (10.59, 68.80) | P = 0.000; Z = 3.727 |
| IFN-γ (pg/ml)  | 33.99 (12.94, 93.03) | 42.48 (26.24, 111.56) | 33.18 (5.02, 93.03) | P = 0.008; Z = 2.636 |
| IL-10 (pg/ml)  | 7.26 (3.08, 17.00) | 5.02 (2.98, 10.13) | 7.92 (3.25, 18.03) | P = 0.012; Z = 2.498 |
| IL-17A (pg/ml) | 12.30 (3.62, 44.74) | 15.66 (9.84, 44.46) | 8.90 (3.34, 45.06) | P = 0.002; Z = -3.037 |
| IL-6 (pg/ml)   | 2.28 (1.10, 9.32) | 3.17 (1.53, 18.33) | 2.26 (0.98, 9.29) | P = 0.061; Z = -1.872 |
| TGFβ1 (pg/ml)  | 2274.50 (3.63, 4751.75) | 4921.50 (3840.75, 7689.50) | 2234 (3.56, 3387) | P = 0.000; Z = -4.404 |
| TGFβ2 (pg/ml)  | 425.81 (348.70, 647.41) | 419.01 (311.80, 580.51) | 435.48 (348.70, 681.08) | P = 0.398; Z = 0.846 |
| TGFβ3 (pg/ml)  | 156.35 (131.69, 198.01) | 131.69 (112.78, 171.94) | 162.61 (135.16, 207.50) | P = 0.025; Z = 2.245 |
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Significantly lower levels of IL-10 and IFN-γ than those with high ALT levels. CHB patients with high ALT have higher NK cell activity, and since NK cells can secrete IFN-γ, CHB patients with high ALT levels have higher IFN-γ levels [20]. There was obvious liver inflammation in CHB patients with high ALT levels. Mononuclear macrophages are important immune cells involved in liver inflammation, and the increased activity of these mononuclear macrophages leads to an increase in the IL-10 level [9, 21].

All cytokines were included in the analysis of risk factors for CHB patients with chronic HBV infection in the immunotolerant phase and chronic hepatitis B. In multivariate logistic regression analysis, differences in TGF-β 1 and TGF-β 2 levels were statistically significant ($P < 0.001$).

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

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

| Cytokine | OR (95%CI) | $P$-value |
|----------|------------|-----------|
| Single-factor logistic regression analysis | | |
| Flt3-L | 1.000 (0.9981-0.001) | 0.715 |
| IFN-α2 | 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-β1 | 1.000 | 0.088 |
| TGF-β2 | 1.000 (0.999-1.001) | 0.889 |
| TGF-β3 | 1.008 (0.999-1.018) | 0.097 |
| Multiple-factor logistic regression analysis | | |
| TGF-β1 | 0.999 (0.999-1.000) | $P < 0.001$ |
| TGF-β2 | 1.008 (1.004-1.012) | $P < 0.001$ |

OR, odds ratio; 95% CI, confidence interval

Fig. 2 Comparison of cytokine levels between the IT and CHB groups. A. Flt3-L. B. IFN-α2. C. IFN-γ. D. IL-10. E. IL-17A. F. IL-6. G. TGF-β1. H. TGF-β2. ns, no significance
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Declarations

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

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