Kinetics of phytohemagglutinin-induced IFN-γ and TNF-α expression in peripheral blood mononuclear cells from patients with chronic hepatitis B after liver transplantation

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AIM: To study the association between host immunity and hepatitis B virus (HBV) recurrence after liver transplantation.

METHODS: Peripheral blood mononuclear cells (PBMC) were isolated from 40 patients with hepatitis B and underwent orthotopic liver transplantation (OLT) before and 2, 4, 8 wk after surgery. After being cultured in vitro for 72 h, the levels of INF-γ and TNF-α in culture supernatants were detected with ELISA. At the same time, the quantities of HBV DNA in serum and PBMCs were measured by real time PCR.

RESULTS: The levels of INF-γ and TNF-α in PBMC culture supernatants decreased before and 2, 4 wk after surgery in turn: INF-γ 155.52±72.32 ng/L vs 14.76±9.88 ng/L vs 13.22±10.35 ng/L, F = 6.946, P = 0.027<0.05; TNF-α 80.839±46.75 ng/L vs 18.59±17.29 ng/L vs 9.758±7.96 ng/L, F = 22.61, P = 0.0001<0.05). The levels of INF-γ and TNF-α were higher in groups with phytohemagglutinin (PHA) than in those without PHA before surgery. However, the difference disappeared following OLT. Furthermore, INF-γ and TNF-α could not be detected in most patients at wk 4 and none at wk 8 after OLT. The HBV detection rate and virus load in PBMC before and 2, 4 wk after surgery were fluctuated (HBV detected rate: 51.4%, 13.3%, 50% respectively; HBV DNA: 3.55±0.674 log(10) copies/mL vs 3.00±0.329 log(10) copies/mL vs 4.608±1.344 log(10) copies/mL, F = 7.582, P = 0.002<0.05). HBV DNA in serum was 4.48±1.463 log(10) copies/mL before surgery and <10^3 copies/mL after OLT except for one with 5.72×10^6 copies/mL 4 wk after OLT who was diagnosed as HBV recurrence. The levels of INF-γ and TNF-α were lower in patients with a high HBV load than in those with a low HBV load (HBV DNA detected/undetected in PBMCs: INF-γ 138.08±72.44 ng/L vs 164.24±72.07 ng/L, t = 1.065, P = 0.297>0.05, TNF-α 80.75±47.30 ng/L vs 74.10±49.70 ng/L, t = 0.407, P = 0.686>0.05; HBV DNA positive/negative: INF-γ 136.77±70.04 ng/L vs 175.27±71.50 ng/L, t = 1.702, P = 0.097>0.05; TNF-α 75.37±43.02 ng/L vs 81.53±52.46 ng/L, t = 0.402, P = 0.690>0.05).

CONCLUSION: The yielding of INF-γ and TNF-α from PBMCs is inhibited significantly by immunosuppressive agents following OLT with HBV load increased, indicating that the impaired immunity of host is associated with HBV recurrence after OLT.

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Key words: Liver transplantation; HBV; Recurrence; PBMC

INTRODUCTION

Liver transplantation is the most effective therapy for end-stage liver diseases. The recurrence of primary diseases becomes the main problem, which impedes the long-term survival rate of patients undergoing liver transplantation despite the improvements of surgery and the perioperative management. Chronic hepatitis B virus (HBV) infection is one of the most common diseases leading to a high morbidity and mortality in Asians. In China, there are more than 30 million people suffering from HBV infection. Despite anti-HBs immunoglobulin therapy, HBV infection recurs in a high proportion of patients with HBsAg positive and serum HBV DNA-negative, chronic liver disease after liver transplantation. Therefore, the recurrence of hepatitis B is the critical issue of liver transplantation in China[1]. It was reported that host immunity is closely related with the prognosis of chronic and acute hepatitis B. Few HBV-specific T cells have been found in peripheral blood mononuclear...
cells (PBMCs) of patients with chronic hepatitis B. Lymphocytes are infected with HBV\(^{[3]}\). However, little is known about the immune condition of HBV-infected patients after orthotopic liver transplantation (OLT). After OLT, the HBV load decreases immediately and dramatically, but HBV hiding in extrahepatic tissues especially in PBMCs can infect the graft again\(^{[3,4]}\). Since the function of PBMCs is an important indication of the host immunity status\(^{[5]}\), we investigated the function of PBMCs in producing INF-\(\gamma\) and TNF-\(\alpha\), and the association between host immunity and HBV recurrence after liver transplantation.

**MATERIALS AND METHODS**

**Patients**

A total of 40 patients with hepatic cirrhosis and hepatocellular carcinoma (HCC) who underwent liver transplantation at Sun-Yat Sen University, Guangzhou, from November 2003 to April 2004 were enrolled. Blood samples were obtained one day before and 2, 4 and 8 wk after surgery.

Immunosuppression consisted of tacrolimus-based/cyclosporin-based dual therapy with prednisolone. The doses were adjusted to maintain desired blood levels (5-15 \(\mu\)g/L tacrolimus, 100-250 \(\mu\)g/L cyclosporin) for the 1st year. Prednisolone was commenced at a daily dose of 60 mg, reduced by 8 mg every 4 d after the 2nd wk, and withdrawn completely at a median of 3 mo. mAbs were used for patients whose ascites >3 000 mL or serum Cr >132.6 \(\mu\)mol/L or were used for those infected with bacteria within 2 wk before surgery. The protocol for prevention of HBV recurrence after liver transplantation was as follows: intra-operative administration of 400 IU hepatitis B immunoglobulin intramuscular injection during the an-hepatic phase, followed by 400 IU intramuscular injection for the first 14 postoperative days, then 400 IU every other day for 2 wk, followed by 400 IU once a week. Immunoprophylaxis was continued indefinitely with monthly administration of 400 IU of HBIG by intramuscular injection. All patients received HBIG and lamivudine (100 mg/d) immunoprophylaxis. HBV recurrence following liver transplantation was defined as the reappearance of HBsAg-HBV DNA in serum and/or positive staining for HBsAg on liver biopsy.

**Isolation and culture of PBMCs**

PBMCs isolated from heparinized venous blood by gradient centrifugation using Ficoll-Hypaque (Shenggong, Ltd, Shanghai, China) were suspended in RPMI-1640 supplemented with 10\% heat-inactivated fetal calf serum (RPMI-1640/10\% FCS) and penicillin-streptomycin (Sigma). A total of 2×10\(^6\) PBMC were added to each well (48-well plates, Invitrogen, Carlsbad, CA, USA) for stimulation with or without 2 pg/L of phytohemagglutinin (PHA-P, Atlanta, GA, USA) at 37 \(^\circ\)C for 72 h. All supernatants were collected and stored at -20 \(^\circ\)C until use.

**Measurement of cytokine concentration by ELISA**

Immunoreactive IFN-\(\gamma\) and TNF-\(\alpha\) levels in PBMCs before and after surgery fluctuated (HBV detectable rate: 52.5\% (21/40) of patients before surgery. Almost all patients had an undetectable HBV DNA level (<10\(^6\) copies/mL), except for one patient who had a detectable HBV DNA level at wk 4 after surgery (5.72×10\(^6\) copies/mL). Recurrence of hepatitis B was confirmed by an immunohistochemistry of liver biopsy in this patient.

**HBV DNA in PBMCs**

The HBV detectable rate and HBV DNA load in PBMCs before and after surgery varied. The HBV detectable rate before and after surgery was 51.4\%, 13.3\%, 50\% respectively; HBV DNA: 3.55±0.674 vs 3.00±0.329 vs 4.608±1.344, \(F=7.582, P=0.002\).
Table 1 IFN-γ levels in PBMC culture supernatants at different time points

| Source                     | DF  | SS         | MS   | F       | P   |
|----------------------------|-----|------------|------|---------|-----|
| Factor (pre−2 wk/4 wk)     | 2   | 28 770.02  | 14 385.01 | 6.946  | 0.027 |
| Factor treat               | 2   | 27 072.82  | 13 536.409 | 6.536  | 0.031 |
| Error                      | 6   | 12 425.41  | 2 070.902  |        |      |
| Treat(PHA+/−)              | 1   | 26 999.63  | 26 999.63  | 10.269 | 0.049 |
| Partly factor              | 1   | 13 665.74  | 13 665.74  | 5.169  | 0.107 |

Table 2 TNF-α levels in PBMC culture supernatants at different time points

| Source                     | DF  | SS         | MS   | F       | P   |
|----------------------------|-----|------------|------|---------|-----|
| Factor (pre−2 wk/4 wk)     | 1.233 | 32 186.69 | 26 113.60 | 22.612 | 0.0001 |
| Factor treat               | 1.233 | 3 330.98  | 2 702.48  | 2.340  | 0.1350 |
| Error                      | 38  | 27 045.63 | 1 154.87  |        |      |
| Treat(PHA+/−)              | 1   | 2 363.21   | 2 363.206 | 3.192  | 0.0900 |
| Partly factor              | 1   | 12 425.41  | 2 070.902 |        |      |

Table 3 HBV DNA in serum and PBMCs (mean±SD)

| HBV DNA in serum (copies/mL) | IFN-γ (ng/L) | t   | P   | TNF-α (ng/L) | t   | P   |
|-----------------------------|--------------|-----|-----|--------------|-----|-----|
| Detected                    | 138.08±72.44 | 1.065 | 0.297 | 80.75±47.30  | 0.407 | 0.686 |
| Undetected                  | 164.24±72.07 |      |     | 74.10±49.70  |      |     |
| >1 000                      | 136.77±70.04 | 1.702 | 0.097 | 75.37±43.02  | 0.402 | 0.690 |
| <1 000                      | 175.27±71.50 |      |     | 81.53±52.46  |      |     |

Effect of HBV DNA on IFN-γ and TNF-α level and PBMC culture supernatants

According to the HBV DNA level in serum and PBMCs before surgery, patients were divided into four groups. HBV DNA was >1 000 and <1 000 copies/mL in two groups respectively. HBV DNA was detected and undetected in the other two groups. Comparing the IFN-γ and TNF-α levels in various groups, we found that the HBV DNA level was higher, the cytokine level was lower (Table 3).

DISCUSSION

Several studies showed that the following factors influence the HBV recurrence after OLT[6-11]; the HBV infection before surgery, the administration of immunosuppressive agents, the HBV level in extrahepatic tissues and the genotype of HBV. It is generally accepted that patients with active replication of HBV before the surgery and on high dose immunosuppressive agents are easier to be reinfected. In addition, the infection of PBMC might lead to the selection of HBV variants which contribute to immunologic escaping[46]. Previous studies showed that the pattern of cytokines produced by circulating PBMCs from patients underwent OLT would determine the immunologic state of transplanted allograft[12]. However, the function of PBMCs of HBV-infected patients who underwent OLT is still unclear.

Cytokines play an important role in antiviral immunity. After infecting the host cells, HBV is eliminated by the host immune system through two pathways[13,14]. One is the cytolytic pathway characterized by activated HBV-specific T cells, mediating the effect of cellular cytotoxicity and lysis of HBV-infected cells. The other is mediated by cytokines, especially by IFN-γ and TNF-α, which depress the replication and expression of HBV, degrade HBV[15-17]. Recently, evidence supports that the non-cytolytic immune-mediated pathway is the principal way to eliminate viruses. Since the function of PBMCs reflects the host immunity to HBV[18], it is useful to evaluate the graft immunity state and the change of host anti-viral immunity through monitoring the function of PBMCs in producing IFN-γ and TNF-α post OLT.

In our study, the levels of IFN-γ and TNF-α in PBMCs culture supernatants decreased dramatically post operation, consistent with literature reports, 50% cytokine reduction under clinical dose of CsA and FK506[19]. Other reports showed that the TNF-α plasma level increased in the 1st wk post OLT[20]. However, we did not detect the cytokine plasma concentrations. Since liver transplantation may lead to changes in the metabolic activity of neutrophils, it is necessary to perform further detailed study about IFN-γ and TNF-α plasma levels.

We also found that there were no differences between the groups with and without PHA, suggesting that PBMCs do not respond to the stimulation of PHA. On the contrary, in Chen’s study[18], the increased IFN-α mRNA expression after stimulated by PHA was reported. TNF-α expression induced by PHA in PBMC was higher in patients with an acute rejection episode. There are several underlined reasons that contributed to the difference between the two studies: first, the patients enrolled in Chen’s study had previous rejection episodes, while in our study none had rejection. Second, in China, most patients who underwent OLT had serious complications and the human mAb of Tac was given to inhibit and then impair the function of T cells completely. Third, FK506 is preferred in our immunosuppressive...
protocol. Sakuma et al., found that compared with CsA and DEX, FK506 may be most effective in specifically preventing T cell activation mediated inflammatory cytokine production in a clinic setting. We are not clear which immunosuppressive agents were favored in Chen’s study.

The present study showed no differences between the groups with and without PHA. It may be because the multi-immunosuppressive agents downregulated the receptors on T cells and fewer signals were transmitted into the cells. However, the function of T cells was partially suppressed in this period and produced cytokines. In clinic, acute rejection occurs within 1 mo and more frequently within 2 wk after surgery, indicating that the cell-mediated immunity is partially depressed during this period. At the same time, HBV DNA in PBMCs decreases, suggesting that it was a relatively safe period to avoid HBV reinflection. Four weeks following surgery, cytokines in culture supernatants cannot be detected in most patients. This may be explained by the following reasons. Firstly, immunosuppressive agents block the activation and proliferation of T cell and the production of cytokines are suppressed. Secondly, these cannot be detected in most patients. This may be explained by the following reasons. Firstly, immunosuppressive agents block the activation and proliferation of T cell and the production of cytokines are suppressed. Secondly, these cannot be detected in most patients.

Four weeks following surgery, cytokines in culture supernatants cannot be detected in most patients. This may be explained by the following reasons. Firstly, immunosuppressive agents block the activation and proliferation of T cell and the production of cytokines are suppressed. Secondly, these cannot be detected in most patients.

At the same time, the detectable rate of HBV DNA in PBMCs increased, and the virus load in PBMCs was near to that before surgery. The HBV DNA level in serum affects the cytokine level in PBMCs. Higher HBV DNA would inhibit the production of IFN-γ induced by IL-12 in chronic hepatitis B patients. The reduction of virus load and antigen would repair the anti-virus capability was the lowest at this time, the HBV DNA level increased, and the virus load in PBMCs was near to that before surgery. The HBV DNA level in serum affects the cytokine level in PBMCs. Higher HBV DNA would inhibit the production of IFN-γ induced by IL-12 in chronic hepatitis B patients. The reduction of virus load and antigen would repair the anti-virus capability was the lowest at this time, the HBV DNA level increased, and the virus load in PBMCs was near to that before surgery. The HBV DNA level in serum affects the cytokine level in PBMCs. Higher HBV DNA would inhibit the production of IFN-γ induced by IL-12 in chronic hepatitis B patients. The reduction of virus load and antigen would repair the anti-virus capability was the lowest at this time, the HBV DNA level increased, and the virus load in PBMCs was near to that before surgery.

In conclusion, the yielding of INF-γ from PBMCs is inhibited significantly by immunosuppressive agents following OLT with HBV load increased, indicating that the impaired immunity of host is associated with HBV recurrence after OLT. The HBV DNA level in serum affects the cytokine level in PBMCs. Higher HBV DNA would inhibit the production of IFN-γ induced by IL-12 in chronic hepatitis B patients. The reduction of virus load and antigen would repair the anti-virus capability was the lowest at this time, the HBV DNA level increased, and the virus load in PBMCs was near to that before surgery.

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