Association of CD4⁺CD25⁺Foxp3⁺ regulatory T cells with chronic activity and viral clearance in patients with hepatitis B

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Abstract

Chronic activity of hepatitis B is thought to involve aberrant immune tolerance of unknown mechanism. In this study, we examined the role of CD4⁺CD25⁺Foxp3⁺ regulatory T cells in disease activity and viral clearance in hepatitis B. Patients with chronic active hepatitis B (CAH) and asymptomatic HBV carriers (AsC) exhibited a significantly high frequency of CD4⁺CD25⁺Foxp3⁺ T cells as opposed to that of controls and resolved HBV infection. These CD4⁺CD25⁺ T cells expressed an elevated level of Foxp3 and displayed increased inhibitory activity towards both CD4⁺CD25⁻ and CD8⁺ effector cells. They were found to accumulate in liver biopsy tissue of CAH patients as opposed to controls. The frequency of CD4⁺CD25⁺Foxp3⁺ T cells correlated positively with hepatitis B envelope (HBe) antigen status and serum HBV DNA copy numbers and had a converse relationship with HBe antibody status in patients with CAH and AsC. It was evident that in these patients, the increased frequency of CD4⁺CD25⁺Foxp3⁺ T cells was associated with serum levels of transforming growth factor-β known to promote peripheral conversion of CD4⁺CD25⁻ T cells to CD4⁺CD25⁺Foxp3⁺ regulatory T cells. The findings provide new insights into the role of CD4⁺CD25⁺Foxp3⁺ regulatory T cells in chronic activity and viral clearance in chronic hepatitis B.

Introduction

Chronic hepatitis B is a significant medical problem worldwide and, in particular, in China where roughly up to 10–20% of the population is persistently infected with hepatitis B virus (HBV) (1–3). In most adult cases (~95%), HBV infection leads to acute hepatitis that runs a self-limiting course with a complete resolution (4, 5). However, in a significant proportion of HBV infection, patients develop chronic hepatitis, and some become HBV carriers. The chronic activity of hepatitis B carries with it significant disease burden and is associated with a risk for cirrhosis and hepatocellular carcinoma (6–9). It has been puzzling for years why some patients develop chronic hepatitis while, in others, HBV infection resolves without clinical consequences and why in chronic carriers viral infection is not cleared. Although these issues are unresolved, there has been speculation that aberrant immune tolerance in chronic hepatitis and HBV carriers plays an important role in impairing the immune responses critical for viral clearance (10, 11).

Recent advances in the understanding of the properties of CD4⁺CD25⁺ regulatory T cells have provided new insights into the mechanism by which immune tolerance is maintained or broken in various disease conditions (12–14). Significant deficits in the number and function of these regulatory T cells were found in autoimmune diseases, including multiple sclerosis, in which impaired immune tolerance is blamed for altered anti-tumor immunity or persistent microbial infections.
(15–23). These studies have demonstrated the importance of CD4\(^+\)CD25\(^+\) regulatory T cells in various immune-related diseases and have provided a clue in exploring the role of CD4\(^+\)CD25\(^+\) regulatory T cells in hepatitis B. In this regard, more recent studies have yielded interesting findings (24, 25). The results collectively indicate that immune tolerance in chronic hepatitis may be associated with CD4\(^+\)CD25\(^+\) regulatory T cells. However, the relevance of their role remains elusive with respect to chronicity, HBV viral clearance and possible mechanisms associated with the induction of CD4\(^+\)CD25\(^+\)Foxp3\(^+\) T cells in viral carriers and in patients with chronic hepatitis. This study was undertaken to characterize in detail CD4\(^+\)CD25\(^+\) regulatory T cells, in relation to their frequency, Foxp3 expression and inhibitory activity in blood and liver biopsy tissues, and their potential association with viral clearance and host immune responses to HBV. The study was performed with a Chinese cohort with well-defined hepatitis B. Furthermore, a preliminary mechanism was sought to provide an explanation for the increased frequency and inhibitory function of CD4\(^+\)CD25\(^+\)Foxp3\(^+\) regulatory T cells in chronic hepatitis. The findings have provided new insights into the understanding of the role of aberrant immune tolerance mediated by CD4\(^+\)CD25\(^+\) regulatory T cells in chronic activity and impaired viral clearance in hepatitis B.

**Methods**

**Patients and specimens**

A total of 65 patients with chronic hepatitis B were included in the study. All patients fulfilled the clinical and laboratory criteria for chronic hepatitis B (26). These patients were divided into a chronic active hepatitis B (CAH) group (n = 44) and an asymptomatic HBV carrier (AsC) group (n = 21). An additional group of individuals with resolved HBV infections (resolved, n = 14) was included as control. The inclusion criteria for resolved subjects included a history of HBV infection, normal liver biochemistry, undetectable clinical activity and HBV DNA as well as HBs antigen and hepatitis B envelope (HBe) antigen seroconversion for at least 6 months prior to sample collection. For normal control, PBMCs and sera were obtained from a group of 16 healthy individuals matched for sex ratio and mean age with the patient groups. The clinical data of the patients are summarized in Table 1.

Not all samples were analyzed for each of the indicated assays because of limited cell numbers. Patients with suspected co-infection or superinfection with HIV and other hepatitis viruses (types A, C, D and E) were excluded from this study. Patients with an overt co-morbid condition such as immunocompromised disease and autoimmune disease, and patients that received anti-viral (IFNs or nucleotide analog), immunomodulatory or immunosuppressive treatments during the last 6 months before the sample collection were all excluded. Liver biopsy tissue specimens were taken by needle puncture in a separate setting (unrelated to this study) for diagnostic purposes. The study protocol and consent form were approved by the institutional review board of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

**Flow cytometry**

PBMCs were re-suspended in PBS containing 1% BSA (Sigma–Aldrich, St Louis, MO, USA) and 0.1% sodium azide. For surface staining of CD4, CD25 and glucocorticoid-inducible tumor necrosis factor receptor (GITR), PBMCs were incubated with fluorochrome-conjugated antibodies to the indicated cell surface markers (eBioscience, San Diego, CA, USA) at the recommended dilution or with isotype control antibodies for 30 min at 4°C. For intracellular staining of Foxp3 and CTLA-4, cells were fixed and permeabilized with Foxp3 staining buffer (eBioscience). Resulting cells were stained with PE-conjugated antibodies to Foxp3 (20 µl per 10^6 cells, clone PCH101 Set, eBioscience) or CTLA-4. Stained cells were analyzed subsequently using a FACSArıa\(^{TM}\) instrument (Becton Dickinson, San Jose, CA, USA).

**Preparation of CD4\(^+\)CD25\(^+\) regulatory T cells**

Fresh PBMCs were used for the isolation of CD4\(^+\)CD25\(^+\), CD4\(^+\)CD25\(^−\) and CD8\(^+\) T cells. CD4\(^+\) T cell populations were isolated from PBMCs by negative selection using a CD4\(^+\) T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). CD4\(^+\)CD25\(^+\) T cells were further isolated from CD4\(^+\) T cells using microbeads (Miltenyi Biotec) according to the manufacturer’s instructions. CD8\(^+\) T cells were positively selected using magnetic beads coated with anti-human CD8 antibody (Dynal Biotech ASA, Oslo, Norway) and subsequently recovered by the addition of CD8 detachbeads (Dynal Biotech). In some experiments, CD4\(^+\)CD25\(^+\) T cells were FACS sorted using a FACSArıa\(^{TM}\) instrument (Becton

<table>
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<th>Table 1. Clinical characteristics and laboratory findings</th>
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<td><strong>Normal subject</strong></td>
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</tr>
<tr>
<td>Number of cases</td>
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<td>Sex (M/F)</td>
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<td>ALT (U l(^{-1}))</td>
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<td>AST (U l(^{-1}))</td>
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<td>HBeAg positive</td>
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<td>HBeAb positive</td>
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<td>HBV DNA copy number (x10^7 ml(^{-1}))</td>
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ALT: alanine aminotransferase (normal range: 5–40 IU l\(^{-1}\)); AST: aspartate aminotransferase (normal range: 5–40 IU l\(^{-1}\)); HBeAg: hepatitis B envelope antigen and HBeAb: hepatitis B envelope antibody.
Dickinson). The purity of CD4+CD25+, CD4+CD25- or CD8+ T cell fractions was always >95%.

Detection of Foxp3 expression by real-time PCR
Total RNA was isolated from CD4+CD25+ T cells using RNeasy Mini Kit (Qiagen, Hilden, Germany) and first-strand cDNA was subsequently synthesized using Sensiscript RT Kit (Qiagen) according to the manufacturer’s instructions. mRNA expression of Foxp3 was determined by real-time PCR using SYBR Green master mix (Applied Biosystems, Foster City, CA, USA). Thermocycler conditions included an initial holding at 50°C for 2 min and subsequently at 95°C for 10 min, which was followed by a two-step PCR program consisting of 95°C for 15 s and 60°C for 60 s for 40 cycles. Data were collected and quantitatively analyzed on an ABI Prism 7900 sequence detection system (Applied Biosystems). The human GAPDH gene was used as an endogenous control for sample normalization. Results were presented as fold relative to the expression of GAPDH. Sequences of PCR primer pairs were as follows: human Foxp3, forward 5'-CGGACCATCTTCTGGATGAG-3' and reverse 5'-TTGTCGGATGATGCCACAG-3', and human GAPDH, forward 5'-GAAGGTGAAGGTGACGTC-3' and reverse 5'-GAAGATGTGATGAGGATTC-3'.

Immunohistochemistry
Liver biopsy tissues were obtained from procedures performed for other medical reasons. Tissues were fixed in buffered formalin and embedded in paraffin using standard procedures. Antibody staining of paraffin sections was performed by immunohistochemistry using deparaffinized retrieval solution (Zhongda Institute of Medical Application, Shanghai, China). Endogenous peroxidase was blocked prior to staining. Slides were incubated at 4°C overnight with a mAb to Foxp3 (clone 236A/E7, courtesy of Giovanna Roncador; ref. 27). Detection was performed with a biotin-conjugated goat anti-mouse secondary antibody (Boster Biological Technology, Wuhan, China) followed by peroxidase-labeled streptavidin (Boster Biological Technology) and diaminobenzidine chromogen (Boster Biological Technology) as a substrate. Sections were counterstained with hematoxylin. Staining with secondary antibody in the absence of Foxp3 antibody was used as a negative control. Foxp3+ T cells were enumerated microscopically in five randomly selected inflammatory areas per sample by two independent operators.

Inhibitory activity of CD4+CD25+ regulatory T cells
To evaluate the inhibitory activity, freshly isolated CD4+CD25+ or CD8+ T cells were used as responders. CD4+CD25+ T cells (2 × 10^4 per well) were stimulated with plate-bound anti-CD3/CD28 antibodies (2 µg ml^-1, ebioscience) and irradiated autologous PBMCs as antigen-presenting cells (10^5 per well) in the presence or absence (naïve CD4+CD25- T cells alone) of CD4+CD25+ T cells used at the cell density of 2 × 10^5 per well. The ability of CD4+CD25+ T cells (inhibitor) to suppress the proliferation of purified CD4+CD25- or CD8+ T cells (responder) was determined by [3H]thymidine incorporation. Percentage of inhibition on the proliferation of responder was calculated as [1 – (experimental counts per minute/control counts per minute)] × 100%.

Measurement of serum transforming growth factor-β by ELISA
Serum levels of transforming growth factor-β (TGF-β) were measured using a human TGF-β1 ELISA kit (Jingmei Biotech, Shenzhen, China). Briefly, microtiter plates were washed and treated with 10% FCS for 1 h to saturate non-specific binding sites. Serum specimens were diluted and added in PBS–Twee 20 in duplicate wells. Plates were incubated for 2 h and subsequently washed with PBS–Twee 20. Monoclonal detecting antibody (Jingmei Biotech) was added and incubated for additional 2 h. After washing, HRP-conjugated goat anti-mouse IgG pre-absorbed against human IgG (Southern Biotechnology Associates, Birmingham, AL, USA) was used prior to color development with 3,3',5,5'-tetramethylbenzidine. Optical density was measured and calculated using an ELISA reader equipped with specialized software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistics
Differences in the expression of genes between the groups were analyzed by the Mann-Whitney U-test. One-way analysis of variance was initially performed to determine whether an overall statistically significant change existed before using the two-tailed paired or unpaired Student's t-test. A P value of <0.05 was considered statistically significant.

Results
Association of the frequency of CD4+CD25+Foxp3+ regulatory T cells with CAH and HBV carriers
Percentage of CD4+CD25+ T cells was initially analyzed in blood specimens obtained from patients with CAH and AsC, along with resolved hepatitis and healthy controls. The results indicated a high frequency of CD4+CD25+ T cells in patients with CAH and AsC (data not shown). However, it was noted that not all CD4+CD25+ T cells in these patients represented regulatory T cells and that chronic HBV infection and liver inflammation could lead to circulation of activated T cells that also express the CD4+CD25+ phenotype. Thus, we determined the frequency of CD4+CD25+ regulatory T cells by measuring CD4+CD25+ T cells that expressed Foxp3, a transcription factor commonly used for the identification of CD4+CD25+ regulatory T cells (28–30). As shown in Fig. 1(A and B), the frequency of CD4+CD25+ T cells co-expressing Foxp3 increased significantly in the blood of patients with CAH and AsC compared with that of controls as measured by intracellular staining by flow cytometry (P < 0.05). Consistent with intracellular staining was markedly increased mRNA expression of Foxp3 in purified CD4+CD25+ T cell populations obtained from the same patients (Fig. 1C). The expression of Foxp3 in the CD4+CD25+ T cell population appeared to correlate with that of GITR, another marker associated with CD4+CD25+ regulatory T cells, but not CTLA-4 (Fig. 1D). We then investigated the frequency of Foxp3+ T cells in liver biopsy tissues obtained from patients with CAH and AsC. As illustrated in Fig. 2, Foxp3+ cells were present in significantly higher numbers in liver tissue sections
from CAH and, to a lesser degree, in AsC, as opposed to a case of acute hepatitis B and four cases of non-alcoholic steatohepatitis.

Regulatory activity of CD4+CD25+ regulatory T cells and association with viral clearance

CD4+CD25+ T cells were purified from patients and examined for their regulatory activity on both CD4+ and CD8+ effector cells. The results showed that purified CD4+CD25+ T cells from patients with CAH and AsC exhibited a moderately increased inhibitory activity toward the proliferation of both CD4+CD25+ T cells and CD8+ T cells of the same patients as a source of target cells (Fig. 3). We further addressed whether the frequency of CD4+CD25+Foxp3+ T cells correlated with serum HBV DNA copy numbers and the HBe antigen status indicative of HBV infectivity and its serum antibody among patients with CAH and AsC. The results revealed that the increased frequency of CD4+CD25+Foxp3+ T cells correlated positively with the HBe antigen status and conversely with the HBe antibody status ($P < 0.05$, Fig. 4A). Moreover, there was significant correlation between the frequency of CD4+CD25+Foxp3+ T cells and serum DNA copy numbers of HBV ($P = 0.003$, Fig. 4B). The findings indicate that the frequency of CD4+CD25+Foxp3+ T cells appears to correlate with chronic clinical activity of hepatitis B and with impaired viral clearance.

Association of increased concentrations of serum TGF-β and CD4+CD25+ T cells in CAH and AsC

It was of great interest to explore possible mechanisms underlying aberrant frequency and inhibitory function of CD4+CD25+ T cells in patients with CAH and AsC. In this regard, serum TGF-β levels were measured to determine whether they correlated with the frequency and Foxp3 expression of CD4+CD25+ regulatory T cells in CAH and AsC patients as TGF-β has been shown to promote the conversion of CD4+CD25+ T cells to CD4+CD25+Foxp3+ regulatory T cells through the induction of Foxp3 expression (31–34). Our study revealed that serum concentrations of TGF-β were significantly increased in patients with CAH ($P < 0.01$) and, to a lesser extent, in patients with AsC ($P < 0.05$) (Fig. 5A). Moreover, serum levels of TGF-β correlated significantly with
the frequency of CD4^CD25^Foxp3^+ T cells and mRNA expression of Foxp3 in purified CD4^CD25^+ T cells in these patients (Fig. 5B and C). Collectively, the findings support our hypothesis that the increased frequency and aberrant function of CD4^CD25^+ regulatory T cells seen in patients with CAH and AsC may be attributable, at least in part, to the high production of TGF-β in a chronic state of hepatitis B. However, at this time, another possibility that HBV-encoded
viral antigens directly induce the expression of Foxp3 expression and conversion of CD4^+CD25^+ regulatory T cells cannot be completely ruled out and warrants further investigation.

**Discussion**

Impaired viral clearance and chronic active state of hepatitis B have been linked to aberrant immune tolerance (10, 11). However, the exact relationship between clinical pathology of CAH and mechanism of inadequate immune tolerance remains elusive. The study presented here provides new insights into the role of CD4^+CD25^+ regulatory T cells in aberrant immune tolerance and potential mechanisms whereby CD4^+CD25^+ regulatory T cells are up-regulated in vivo in patients with chronic activity of the disease. The study represents an important example of the manner in which CD4^+CD25^+ regulatory T cells are involved in human pathological conditions, in addition to their role in autoimmune...
diseases and cancers (15–17). First, we demonstrated that the frequency and function of CD4^+CD25^+ regulatory T cells are elevated in patients with chronic active hepatitis. It was evident that these CD4^+CD25^+ regulatory T cells are accumulated in the liver and that the frequency of both peripheral and intra-hepatic regulatory T cells correlated with the chronic state of hepatitis B. Consistent with the findings described here are those reported recently by Stoop et al. (24) who showed increased frequency of CD4^+CD25^+ regulatory T cells in a Dutch cohort of chronic hepatitis B. Our results, however, not only indicate that such regulatory T cells accumulated at the site of pathology but also demonstrate a significant relationship between the status of HBe antigen and antibody and the levels of the frequency, Foxp3 expression and the inhibitory function of CD4^+CD25^+ regulatory T cells.

Another important aspect of the study is related to the mechanism potentially involved in the persistent induction of Foxp3 over-expression and the function of regulatory T cells. There are several possibilities that may explain aberrant frequency and function of CD4^+CD25^+ regulatory T cells. For example, the gene products of HBV, such as the envelope proteins, may directly induce CD4^+CD25^+Foxp3^+ regulatory T cells through antigen recognition. Similar situations with TCR peptides or TLR2 in the induction of CD4^+CD25^+ regulatory T cells have been reported (35–37). However, a more likely mechanism is related to certain cytokine that is characteristically over-produced in the context of CAH. Such a cytokine may promote peripheral conversion of CD4^+CD25^+ T cells to CD4^+CD25^+ regulatory T cells. In this regard, TGF-β seems to meet the two requirements for the induction of Foxp3 expression in hepatitis B. First, it has been shown to have a unique property in inducing such a conversion (31–34). Second, TGF-β has been found to occur at an increased concentration in both sera and hepatic tissues in patients with CAH (38–40). Indeed, our results presented here indicate that there is a significantly elevated serum level of TGF-β compared with that of controls and that TGF-β serum levels correlate significantly with the expression of Foxp3 and the frequency and function of CD4^+CD25^+ regulatory T cells in chronic hepatitis patients. Taken together, the findings provide an explanation, at least in part, for aberrant frequency and function of CD4^+CD25^+ regulatory T cells seen in patients with chronic hepatitis.

Acknowledgements

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Abbreviations

AsC asymptomatic HBV carrier
CAH chronic active hepatitis B
GITR glucocorticoid-inducible tumor necrosis factor receptor
HBe hepatitis B envelope

Fig. 5. Serum concentrations of TGF-β and correlation with the frequency of CD4^+CD25^+Foxp3^+ T cells and the expression levels of Foxp3 in CD4^+CD25^+ T cells. (A) Serum concentrations of TGF-β were measured in patients with CAH (n = 44), AsC (n = 17), resolved (n = 14) and healthy controls (n = 16) by ELISA. Serum concentrations of TGF-β were correlated with the frequency of CD4^+CD25^+Foxp3^+ T cell (B) and mRNA expression levels of Foxp3 in purified CD4^+CD25^+ T cells (C) from CAH (n = 19) and AsC (n = 21) patients. Asterisks indicate statistically significant differences between the groups (P < 0.05).
References