CASE REPORT

Immunological Analysis of a Patient with Hepatitis B Virus (HBV) Reactivation after Bone Marrow Transplantation

Yuka Kowazaki¹, Yosuke Osawa¹, Jun Imamura¹, Kazuteru Ohashi², Hisashi Sakamaki² and Kiminori Kimura¹

Abstract

Patients with resolved hepatitis B virus (HBV) infection undergoing chemo- or immunosuppressive therapy are at potential risk for HBV reactivation. To determine whether the host immune response contributes to liver injury, we performed an immunological analysis of a patient with HBV reactivation. Consistent with the detection of HBV DNA in the sera, the number of polyclonal HBV-specific cytotoxic T lymphocytes (CTLs) gradually increased; however, the number of CD4⁺CD25⁺ regulatory T cells (Treg) decreased. The interaction between HBV-specific CTLs and CD4⁺CD25⁺ Treg is an important determinant of liver injury during HBV reactivation. Therefore, monitoring the number of these cells might be a useful modality for the diagnosis of acute hepatitis resulting from HBV reactivation.

Key words: HBV reactivation, CTL, regulatory T cell, FACS

(Intern Med 54: 1213-1217, 2015)
(DOI: 10.2169/internalmedicine.54.3706)

Introduction

More than 300 million people worldwide suffer from chronic hepatitis B virus (HBV) infection, and it is a common cause of morbidity and mortality (1, 2). An estimated one million people die annually from complications associated with chronic HBV infection, such as cirrhosis, end-stage liver disease, and hepatocellular carcinoma (3, 4).

HBV reactivation is a well known phenomenon that commonly occurs in hepatitis B surface antigen (HBsAg) carriers undergoing immunosuppressive therapy. Some immunosuppressive therapies may enhance HBV replication in hepatocytes, which results in detectable levels of serum HBV DNA, followed by clinical hepatitis (1). The development of HBs and hepatitis B core antigen (HBcAg) antibodies in conjunction with the loss of HBsAg following acute HBV infection is thought to represent clearance of the virus. However, increasing evidence suggests that the virus may remain latent within the liver (5, 6). The clinical course and disease outcome of HBV infection is modulated by the host immune response (7), and the loss of immune surveillance can cause the reactivation of HBV replication and the exacerbations of liver disease. Reactivation of HBV is a well-characterized syndrome that is marked by the abrupt reappearance of or increase in HBV DNA in the serum of a patient with previously inactive or resolved HBV infection (1, 8). The mechanism responsible for the reactivation of the virus and the associated liver damage may include a rebound in the number of lymphocytes following the cessation of immunosuppressive therapy and myelosuppressive chemotherapy. This sudden increase in lymphocytic cells may result in the rapid destruction of infected hepatocytes with consequent severe hepatitis and increased HBV-DNA levels. Fulminant liver failure following HBV reactivation is associated with the HBV genotype Bj, which exhibits a high replication rate owing to the A1896 mutation (9). Thus, although there is evidence that viral factors are involved in the mechanism driving HBV reactivation, it remains unknown precisely how the host immune response affects liver injury and the viral load.

In this study, we describe the clinical case of HBV reactivation following bone marrow transplantation. We monitored the antigen-specific immune responses that occurred during

¹Division of Hepatology, Tokyo Metropolitan Cancer and Infectious Diseases Center Komagome Hospital, Japan and ²Division of Hematology, Tokyo Metropolitan Cancer and Infectious Diseases Center Komagome Hospital, Japan

Received for publication July 22, 2014; Accepted for publication September 23, 2014
Correspondence to Dr. Kiminori Kimura, kkimura@cick.jp
Peripheral blood mononuclear cells (PBMCs)

After the PBMCs were isolated using Lymphoprep™ (Axis-shield, Oslo, Norway) the cells were washed twice in phosphate-buffered saline (PBS, Gibco, Auckland, NZ) and then were studied immediately. The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, 100 mg/mL streptomycin, 50 μg/mL gentamicin, and 2 mM L-glutamine (all from Invitrogen) at 37°C in a humidified 5% CO2 incubator, as previously described (10).

Synthetic peptides

Three HBV peptides, HLA-A*0201 core 18-27 (FLPSDFFPSV), HLA-A*2402 core 117-125 (EYLVSFGVW) and pol 1756-1764 (KYTSFPWLL) were synthesized by Sigma Aldrich (Ishikari, Japan).

MHC-class I tetramer staining

The PE-labeled HLA-A*0201-restricted HBV core (FLPSDFFPSV) and HLA-A*2402 HBV core (EYLVSFGVW) HBV polymerase (KYTSFPWLL) were used in this study (MBL, Nagoya, Japan).

Fluorescence-activated cell sorting (FACS) analysis

To examine the cytokine production of PBMCs, isolated PBMCs were activated with phorbol 12-myristate 13-acetate (PMA, final concentration 10 ng/mL) and then incubated for 4 hours at 37°C in RPMI medium containing 10% fetal bovine serum (Life Technologies, Rockville, USA) and brefedlin A (final concentration: 10 μg/mL) (BD Pharmingen, San Jose, USA). The cells were then stained with anti-CD8, anti-CD4, anti-CD19 and anti-CD25 antibodies (all from BD Pharmingen). After fixation, the cells were permeabilized for 30 minutes in 25 μL of PBS with 0.5% saponin. Anti-human fluorescein isothiocyanate (FITC)-conjugated interferon (IFN)-γ, tumor necrosis factor (TNF)-α, or interleukin (IL)-10 (BD Pharmingen) antibodies were added at a final dilution of 1:100, and the cells were incubated for 30 minutes at room temperature. Intracellular staining was performed using the Foxp3 Staining Buffer Set (eBioscience, San Diego, USA), according to the manufacturer’s instructions. The cells were washed and resuspended in 1 mL of FACS buffer in preparation for analysis using the FACSCanto II system (BD Pharmingen).

Cytokine and chemokine profiles

We used the Bio-Plex cytokine assay kits (Bio-Rad Laboratories, Hercules, USA) to measure the concentrations of cytokines and chemokines in the patient’s sera, following the manufacturer’s instructions. Specifically, the Bio-Plex human cytokine 17-Plex Panel was used, which includes 17 cytokines [IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (P70), IL-13, IL-17, G-cerebrospinal fluid (CSF), GM-CSF, IFN-γ, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1β, and TNF-α]. The plates were then washed three times using vacuum filtration with 100 mL of Bio-Plex wash buffer. Next, 25 μL of diluted detection antibody was added, and the plates were incubated with shaking for 30 minutes at room temperature. After three filter washes, 50 μL of streptavidin-phycoerythrin was added, and the plates were incubated with shaking for another 10 minutes at room temperature. Finally, the plates were washed by vacuum filtration three times, beads were suspended in Bio-Plex assay buffer, and then the samples were analyzed on a Bio-Rad 96-well plate reader using the Bio-Plex Suspension Array System and the Bio-Plex Manager software program (Bio-Rad Laboratories).

Case Report

At the time of pretransplantation screening, a 61-year-old male patient with acute myeloid leukemia (AML) was positive for the HBV-related markers, anti-HBc and anti-HBe antibodies, and negative for HBsAg, anti-HBs, hepatitis B e antigen (HBeAg), and HBV-DNA. In June 2009, he underwent bone marrow transplantation from an HLA matched donor who was negative for HBsAg, anti-HBs and anti-HBc antibodies. In the months following transplantation, the patient was given aggressive immunosuppressive therapy, including prednisone and cyclosporine (CsA) for severe graft-versus-host-disease involving the skin and intestines. At this time, he tested negative for HBsAg, anti-HBs and anti-HBc antibodies. However, five months later, laboratory results showed elevated levels of liver injury markers [aspartate transaminase levels, 143 U/L; alanine transaminase levels, 164 U/L], but a normal international normalized ratio, albumin levels, and total bilirubin levels. Hepatitis B serological tests revealed a newly detectable and high level of HBV DNA (8.6 Log copy/mL). Following these findings, Entecavir treatment was started on the same day. Subsequently, ALT levels decreased gradually and returned to normal levels within 2 months. HBV-DNA titers also decreased gradually to 2.8 Log/mL, until 6 months after Entecavir treatment (Fig. 1).
**HBV-specific CD8+ T cell response during HBV reactivation**

It is well accepted that antigen specific cytotoxic T lymphocytes (CTLs) induce HBV-mediated liver diseases (7). To determine the frequency of HBV-specific CD8+ T cells during HBV reactivation, we isolated PBMCs from the patient and analyzed them with three tetramer specific antibodies for dominant HLA-A2-restricted core-, A24-restricted core- and polymerase-derived epitopes. As shown in Fig. 2A, the number of three tetramer-positive CD8+ T cells increased at every time point as compared with the data obtained at the onset of HBV reactivation. Subsequently, HBV DNA levels also declined, even though the administration of Entecavir had already started. In contrast, the increase in serum ALT levels was delayed by two weeks behind the observed peak in CTL numbers These data indicate that the increase in liver damage and the peak in the number of CTLs are therefore separated temporally, as previously described (11). Furthermore, we assessed the functional properties of the different HBV-specific CD8+ T-cell populations by determining the intracellular cytokine production against the peptide HLA-A2-restricted core-, A24-restricted core- and polymerase-derived epitopes. IFN-γ production against the A2 and A24 core peptides was increased and reached peak levels that corresponded with the number of each tetramer positive cell (Fig. 2B).

**The number of CD4+ regulatory T cells during HBV reactivation**

The levels of circulating CD4+CD25+ Treg have been previously correlated with the viral load in the serum from chronic severe hepatitis B patients (12). To determine whether CD4+CD25+ and CD4+Foxp3+ Treg contribute to liver injury during HBV reactivation, their numbers were monitored in patient PBMCs. As shown in Fig. 2C, the number of CD4+CD25+ or CD4+Foxp3+ Treg gradually declined and reached the lowest point concurrent to when the CTL frequency increased, thus indicating that these cell populations exhibited reversed kinetics.

**Cytokine production of CD8+ T cells and CD19+ B cells**

The HBV reactivation rate was previously found to be increased in patients treated with rituximab-containing chemotherapy (13). This finding suggested that the B cell functions might contribute to HBV reactivation. To determine whether cytokine production by B cells was involved in liver injury, we stained B cells (CD19+) and CD8 T cells with antibodies against IFN-γ, IL-10 and TNF-α using an intracellular cytokine detection method. As shown in Fig. 3, the elevated production of TNF-α and to a lesser extent, IL-10, by CD8 T cells closely correlated with the observed peak in the ALT serum levels. Following a decrease in ALT levels, these cytokines were no longer detected. In contrast,
IL-10 production by CD19+ B cells was increased at the peak serum of ALT levels; however, IFN-γ and TNF-α production was not elevated at this time point.

**Serum levels of inflammatory cytokines and chemokines**

The serum levels of cytokines and chemokines were measured at various time points. As shown in Fig. 4, the serum IL-6, IL-8 and IL-12 p70 levels gradually increased over time, consistent with the observed increase in the serum ALT levels. IL-12 p70 levels peaked one month later than the peak ALT level. Furthermore, the serum MIP-1β levels demonstrated similar kinetics with the ALT levels, thus indicating that the MIP-1β levels might reflect the hepatitis activity.

**Discussion**

HBV reactivation is a serious clinical problem among patients with resolved HBV infection undergoing bone marrow transplantation (14-16). In retrospective analyses using HBV serological markers, a high frequency of anti-HBc cases without HBsAg developed detectable HBV DNA and HBsAg levels following bone marrow transplantation (17-19). In this study, we showed that during HBV reactivation the frequency of antigen-specific CTLs increased, along with functional CTLs that produced IFN-γ against the HBV peptide. These findings are consistent with a previous report that observed a strong, multifaceted CTL response in patients with acute hepatitis (7). In the current case report, following bone marrow transplantation the patient received CsA to prevent graft-versus-host-disease. However, it has been demonstrated that CsA inhibits several Ca2+-dependent transcription factors, including NF-AT, NF-κB, and IL-2 (20). Therefore, it is important to evaluate the proliferation and cytokine production of antigen-specific CTLs during immunosuppressive drug treatment.

In the current study we found that following HBV reactivation, the frequency of antigen-specific CTLs and CD4+ CD25+ Treg was reversed. This implies that a reduction in the number of CD4+CD25+ Treg triggered the induction in antigen-specific CTLs numbers. Although the effect of CD4+ CD25+ Treg inhibition is generally considered nonspecific or bystander, preferential inhibition of Ag-specific T cell responses was observed in some cases, including in human HBV infection (21, 22). In support of our results, Xu et al. demonstrated that CD4+CD25+ Treg depletion led to an increase in the HBV Ag-stimulated production of IFN-γ and the cell proliferation of PBMCs isolated from HBV-infected patients (12). Furthermore, coculture of CD4+CD25+ Treg with effector cells significantly suppressed HBV Ag-stimulated IFN-γ production and cellular proliferation (12).

We also found that the serum MIP-1β concentrations correlated with the serum ALT levels. It was reported that the CC chemokines MIP-1β and regulated on activation, normal T cell expressed and secreted (RANTES) play an important role in hepatic immune surveillance because they are expressed on the portal vessel endothelium and trigger invasion of macrophages and lymphocytes into the liver (23). These chemokines bind to their corresponding receptors, CCR1 and CCR5, both of which are preferentially expressed...
on lymphocytes with a T helper (Th) 1 cytokine secretion pattern, and are critical for viral elimination (24). Although Zeremski et al. showed that the serum MIP-1β levels were elevated in chronic hepatitis C patients, the MIP-1β levels have not yet been examined in patients with HBV reactivation (25). We are currently investigating the role of MIP-1β in the disease activity of hepatitis.

Antigen-specific CTLs produced inflammatory cytokines, such as IFN-γ, that increased at the onset of HBV reactivation, but then gradually returned to baseline levels (Fig. 2B). However, IFN-γ levels peaked when ALT serum levels were normal (Fig. 3B), thus suggesting that the serum and CTL-derived IFN-γ levels should be carefully measured to monitor hepatitis disease activity. Furthermore, the correlation identified between the number of CTLs and HBV reactivation-induced liver damage might also be useful.

In summary, this case report suggests that 1) the reduction of CD4+CD25+ Treg numbers predicts an increase in the antigen-specific CTL and the induction of acute hepatitis and; 2) examining the serum MIP-1β levels may be a potentially useful biomarker for monitoring the hepatitis activity in the future.

The authors state that they have no Conflict of Interest (COI).

Acknowledgement
The authors thank Dr. Michinori Kohara for advice and experimental support. This study was supported by a grant from the Health and Labour Science Research Grants 2014 “Practical Research on Hepatitis” and a grant-in-aid for specially promoted research on viral diseases from the Tokyo Metropolitan Government to K. K.

References
1. Hoofnagle JH. Reactivation of hepatitis B. Hepatology 49: S156, 2009.
2. Raimondo G, Pollincio T, Cacciola I, Squadrito G. Occult hepatitis B virus infection. J Hepatol 46: 160, 2007.
3. Yang JD, Roberts LR. Hepatocellular carcinoma: A global view. Nat Rev Gastroenterol Hepatol 7: 448, 2010.
4. McMahon BJ. The natural history of chronic hepatitis B virus infection. Hepatology 49: S45, 2009.
5. Mason AL, Xu L, Guo L, Kuhns M, Perrillo RP. Molecular basis for persistent hepatitis B virus infection in the liver after clearance of serum hepatitis B surface antigen. Hepatology 27: 1736, 1998.
6. Rehermann B, Ferrari C, Pasquinielli C, Chisari FV. The hepatitis B virus persists for decades after patients’ recovery from acute viral hepatitis despite active maintenance of a cytotoxic T-lymphocyte response. Nat Med 2: 1104, 1996.
7. Chisari FV, Ferrari C. Hepatitis B virus immunopathogenesis. Annu Rev Immunol 13: 29, 1995.
8. Vento S, Cainelli F, Longhi MS. Reactivation of replication of hepatitis B and C viruses after immunosuppressive therapy: an unresolved issue. Lancet Oncol 3: 333, 2002.
9. Sugauchi F, Tanaka Y, Kusumoto S, et al. Virological and clinical characteristics on reactivation of occult hepatitis B in patients with hematological malignancy. J Med Virol 83: 412, 2011.
10. Kimura K, Ando K, Tomita E, et al. Elevated intracellular IFN-gamma levels in circulating CD8+ lymphocytes in patients with fulminant hepatitis. J Hepatol 31: 579, 1999.
11. Guidotti LG, Rochford R, Chung J, Shapiro M, Purcell R, Chisari FV. Viral clearance without destruction of infected cells during acute HBV infection. Science 284: 825, 1999.
12. Xu D, Fu J, Jin L, et al. Circulating and liver resident CD4+ CD25+ regulatory T cells actively influence the antiviral immune response and disease progression in patients with hepatitis B. J Immunol 177: 739, 2006.
13. Yeo W, Chan TC, Leung NW, et al. Hepatitis B virus reactivation in lymphoma patients with prior resolved hepatitis B undergoing anticancer therapy with or without rituximab. J Clin Oncol 27: 280, 2009.
14. Au WY, Lie AK, Liang R, Liu CL, Shek TW, Lau GK. Aggressive hepatocellular carcinoma complicating pregnancy after autologous bone marrow transplantation for non-Hodgkin’s lymphoma. Bone Marrow Transplant 29: 177, 2002.
15. Kojima H, Abei M, Takei N, et al. Fatal reactivation of hepatitis B virus following cytotoxic chemotherapy for acute myelogenous leukemia: fibrosing cholestatic hepatitis. Eur J Haematol 69: 101, 2002.
16. Seth P, Alrajhi AA, Kagevi I, et al. Hepatitis B virus reactivation with clinical flare in allogeneic stem cell transplants with chronic graft-versus-host disease. Bone Marrow Transplant 30: 189, 2002.
17. Kusumoto S, Tanaka Y, Ueda R, Mizokami M. Reactivation of hepatitis B virus following rituximab-plus-steroid combination chemotherapy. J Gastroenterol 46: 9, 2011.
18. Liang R, Lau GK, Kwong YL. Chemotherapy and bone marrow transplantation for cancer patients who are also chronic hepatitis B carriers: a review of the problem. J Clin Oncol 17: 394, 1999.
19. Ustun C, Koc H, Karayalcin S, et al. Hepatitis B virus infection in allogeneic bone marrow transplantation. Bone Marrow Transplant 30: 289, 1997.
20. Shervah EM. The effects of cyclosporin A on the immune system. Annu Rev Immunol 3: 397, 1985.
21. Alatrakchi N, Koziel M. Regulatory T cells and viral liver disease. J Viral Hepat 16: 223, 2009.
22. Manigold T, Racanelli V. T-cell regulation by CD4 regulatory T cells during hepatitis B and C virus infections: facts and controversies. Lancet Infect Dis 7: 804, 2007.
23. Shields PL, Morland CM, Salmon M, Qin S, Hubscher SG, Adams DH. Chemokine and chemokine receptor interactions provide a mechanism for selective T cell recruitment to specific liver compartments within hepatitis C-infected liver. J Immunol 163: 6236, 1999.
24. Sallusto F, Lanzavecchia A, Mackay CR. Chemokines and chemokine receptors in T-cell priming and Th1/Th2-mediated responses. Immunol Today 19: 568, 1998.
25. Zeremski M, Petrovic LM, Talal AH. The role of chemokines as inflammatory mediators in chronic hepatitis C virus infection. J Viral Hepat 14: 675, 2007.

© 2015 The Japanese Society of Internal Medicine
http://www.naika.or.jp/imonline/index.html