Hepatitis B Virus Replication Could Enhance Regulatory T Cell Activity by Producing Soluble Heat Shock Protein 60 From Hepatocytes

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Background. HBcAg-specific regulatory T (Treg) cells play an important role in the pathogenesis of chronic hepatitis B. Soluble heat shock proteins, especially soluble heat shock protein 60 (sHSP60), could affect the function of Treg cells via Toll-like receptor.

Methods. We analyzed the relationship between soluble heat shock protein production and hepatitis B virus (HBV) replication with both clinical samples from HBeAg-positive patients with chronic hepatitis B (n = 24) and HBeAb-positive patients with chronic hepatitis B (n = 24) and in vitro HBV-replicating hepatocytes. Thereafter, we examined the biological effects of sHSP60 with isolated Treg cells.

Results. The serum levels of sHSP60 in patients with chronic hepatitis B were statistically significantly higher than those in patients with chronic hepatitis C (P < .01), and the levels of sHSP60 were correlated with the HBV DNA levels (R = 0.532; P < .001) but not with the alanine aminotransferase levels. Moreover, the levels of sHSP60 in HBV-replicating HepG2 cells were statistically significantly higher than those in control HepG2 cells. Preincubation of CD4+ CD25+ cells with recombinant HSP60 (1 ng/mL) statistically significantly increased the frequency of HBcAg-specific interleukin 10–secreting Treg cells. The frequency of IL7R+ CD4+CD25+ cells, the expression of Toll-like receptor 2, and the suppressive function of Treg cells had declined during entecavir treatment.

Conclusion. The function of HBcAg-specific Treg cells was enhanced by sHSP60 produced from HBV-infected hepatocytes. Entecavir treatment suppressed the frequency and function of Treg cells; this might contribute to the persistence of HBV infection.

Hepatitis B virus (HBV) is a noncytopathic DNA virus that causes chronic hepatitis and hepatocellular carcinoma as well as acute hepatitis and fulminant hepatitis [1]. HBV now affects more than 400 million people worldwide [2], and persistent infection develops in ~5% of adults and 95% of neonates who become infected with HBV.

It has been shown that the cellular immune system, including cytotoxic T lymphocytes, CD4+ T helper 1 cells, and CD4+C25+FoxP3+ regulatory T (Treg) cells, plays a central role in the control of viral infection [3–6]. The hyposresponsiveness of HBV-specific T helper 1 cells and the excessive regulatory function of Treg cells in peripheral blood in patients with chronic hepatitis B has been shown elsewhere [7–10]. Lamivudine treatment of chronic hepatitis B has been reported to restore both CD4+ T cells and cytotoxic T lymphocyte hypo-responsiveness following the decrease of serum levels of HBV DNA and HBV-derived Ag [8, 11–13]. In our previous study, we observed that HBcAg-specific interleukin 10 (IL-10)–secreting Treg cells could play an important role in the immunopathogenesis of chronic hepatitis B [9].
Table 1. Clinical Characteristics of Patients with Chronic Hepatitis B or Chronic Hepatitis C Included in This Study

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients with chronic hepatitis B</th>
<th>Patients with chronic hepatitis C</th>
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<tbody>
<tr>
<td></td>
<td>HBeAg-positive, HBeAb-negative</td>
<td>HBeAg-negative, HBeAb-positive</td>
</tr>
<tr>
<td>Age, years</td>
<td>45.16 (12.46)</td>
<td>48.21 (10.23)</td>
</tr>
<tr>
<td>Sex, no. of patients</td>
<td>Male 12</td>
<td>Male 12</td>
</tr>
<tr>
<td></td>
<td>Female 12</td>
<td>Female 12</td>
</tr>
<tr>
<td>ALT level, IU/L</td>
<td>76.91 (39.82)</td>
<td>75.96 (45.90)</td>
</tr>
<tr>
<td>HBV DNA level, log copies/mL</td>
<td>7.83 (0.86)</td>
<td>6.00 (0.81)</td>
</tr>
<tr>
<td>Genotype, % of patients</td>
<td>A 0</td>
<td>B 4.17</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>8.33</td>
</tr>
</tbody>
</table>

NOTE. Data are mean values (standard deviations), unless otherwise indicated. ALT, alanine aminotransferase; HBV, hepatitis B virus; NA, not applicable.

Many research groups have reported the possible induction of anergy by T_{reg} cells, which constitutively express CD25 (the interleukin 2 receptor α chain) in the physiological state [14–16]. In humans, this population of T_{reg} cells, as defined by CD4^+CD25^-CTLA4^+ cells, CD4^+CD25^-FoxP3^+ cells, or CD4^+CD25^-IL7R^+ cells, constitutes 5%–10% of peripheral CD4^+ T cells and has a broad repertoire that recognizes various self and nonself antigens. It has been reported that T_{reg} cells have several different mechanisms in suppressing various kinds of immune cells [17, 18]. The important mechanisms are cell to cell contact and secretion of cytokines including IL-10 and transforming growth factor β (TGF-β) [19, 20]. HBCAg derived from HBV might induce T_{reg} cells to escape from immunological pressure, as reported in persistent infection with Epstein-Barr virus, hepatitis C virus (HCV), and human immunodeficiency virus type 1 [21–23]. Some results have indicated that reduction of HBV replication could reduce the frequency and/or function of T_{reg} cells in patients with chronic hepatitis B [4, 5, 8]. However, the key factors that affect HBCAg-specific T_{reg} cells in the replication of HBV remain unclear.

The mammalian 60-kDa heat shock protein is a many-faceted molecule. In addition to serving as a chaperone, heat shock protein 60 (HSP60) is expressed by different types of cells following their exposure to stress or immune responses and is present in the blood during inflammation [24–27]. Recently, HSP60 was reported to enhance the function of CD4^+CD25^- regulatory T cell function via Toll-like receptor 2 (TLR2) signaling [28].

In this study, we investigated the serum level of HSP60 in patients with chronic hepatitis B and the relevance of HBCAg-specific IL-10-secreting T_{reg} cells and HSP60. We report evidence of the production of soluble HSP60 (sHSP60) from HBV-replicating hepatocytes, by use of clinical samples from patients with chronic hepatitis B and an in vitro HBV replication system. In addition, reductions of CD4^+CD25^-IL7R^+ T_{reg} cells and TLR2 expression on T_{reg} cells were observed during entecavir therapy. This study could contribute to better understanding of the immunopathogenesis of chronic hepatitis B and the development of immune-based treatment.

MATERIALS AND METHODS

Patients. Forty-eight patients with chronic hepatitis B were enrolled in this study (Table 1). The patients had serum levels of HBV DNA of $>5.0$ log copies/mL and had elevated alanine aminotransferase (ALT) levels (reference range, $<40$ IU/L) for $>6$ months prior to the study. To focus the analysis on the active phase of chronic hepatitis B, we excluded asymptomatic carriers and patients with immune tolerance by age ($<30$ years old), ALT values ($<40$ IU/L), and HBV DNA levels ($<5.0$ log copies/mL). Twenty-four patients were seropositive for HBeAg, and 24 patients were seropositive for anti-HBeAb. None of the patients tested positive for antibodies to hepatitis C virus or had liver disease due to other causes, such as alcohol, drugs, congestive heart failure, and autoimmune disease. Twenty-four patients with chronic hepatitis C and 10 healthy subjects were included as control subjects. Permission for the study was obtained from the Ethics Committee at Tohoku University Graduate School of Medicine (permission no. 2006-194)). Written informed consent was obtained from all the participants enrolled in this study. Participants were monitored for 6 months, and peripheral blood samples were obtained and assessed at 1, 2, 3, and 6 months. At each assessment, patients were evaluated for serum levels of HBV DNA, HBeAg, and anti-HBe, blood chemistry, and hematology. Levels of HbsAg, anti-HBs, total and immunoglobulin anti-Hbc, HBeAg, anti-HBe, and anti-
hepatitis C virus were determined by means of commercial enzyme immunoassay kits (Abbott Laboratories). Serum levels of HBV DNA were measured by means of an Amplicor polymerase chain reaction (PCR) assay (lower limit of detection, 2.6 log copies/mL; Roche). High titers of HBV DNA were measured by means of a transcription-mediated amplification-hybridization protection assay (TMA; lower limit of detection, 3.7 log genome equivalents per milliliter). Data were adjusted by means of the following formula: Amplicor value = 0.83 × (TMA value) + 0.67.

Reagents. The following antibodies were used: CD3–allophycocyanin (APC), CD4–peridinin chlorophyll protein complex (PerCP), CD25–fluorescein isothiocyanate (FITC), CD25–phycoerythrin (PE), CD127–PE, Alexa Fluor 488 mouse anti-human CD282 (TLR2), CD284 (Toll-like receptor 4 [TLR4]), and isotype-matched control antibodies purchased from BD Bioscience. Recombinant HBCAg was obtained from Biodesign International. Recombinant HSP60 (rHSP60) was purchased from Stressgen.

Quantification of sHSP60 and soluble heat shock protein 70 (sHSP70) levels. Levels of HSP60 and heat shock protein 70 (HSP70) were quantified by use of HSP60 and HSP70 enzyme-linked immunosorbent assay (ELISA) kits (Stressgen). The serum samples from patients and supernatants from cell cultures were collected at sampling points and stocked at −20°C. The ELISA procedure was performed according to the manufacturer’s protocol. First, 100-μL prepared samples were added to wells of anti-HSP60-coated plates. Then the reaction of the anti-HSP60 and horseradish peroxidase conjugate was performed after incubation and washing. Absorbance was measured at 450 nm. The HSP60 sample concentration was calculated by use of a standard curve.

Isolation of peripheral blood mononuclear cells (PBMCs) and Treg cells. PBMCs were isolated from fresh heparinized blood by means of Ficoll-Hypaque density gradient centrifugation. Treg cells were isolated by use of a Dynabeads regulatory CD4+CD25+ T cell kit (Invitrogen). Treg cells were isolated according to the manufacturer’s protocol. In brief, CD4+ cells were isolated from PBMCs by means of negative selection. The remaining cells included the PBMCs depleted of CD4+ cells. Then the CD4+CD25+ cells were selected positively by use of CD25 antibody combined with beads. Finally, the beads were detached by means of Detachabead (Invitrogen), because the function of Treg cells might be modified by anti-CD25 antibody.

Coculture of γ-irradiated HBCAg-presenting antigen-presenting cells (APCs) and Treg cells. During the isolation of Treg cells, PBMCs depleted of CD4+ cells could be obtained for use as APCs. PBMCs depleted of CD4+ cells were stimulated at 1 × 10^6 cells/mL in Roswell Park Memorial Institute 1640 medium containing 10% fetal bovine serum with HBCAg (10 μg/mL) for 12 h at 37°C. Then these γ-irradiated cells were coincubated with 1 × 10^5 isolated Treg cells that were untreated pretreated with TLR2 and TLR4 neutralizing antibody and rHSP60 (1 ng/mL) (Figures 1A and 2).

IL-10 secretion assay. Isolated Treg cells were stimulated with HBCAg-presenting autologous γ-irradiated APCs for 12 h at 37°C. IL-10-secreting cells were stained by adding 10 μL of IL-10-detection antibody (PE-conjugated) together with anti-CD4-PerCP, anti-CD25-FITC, and anti-CD3-APC.

Flow cytometry. PBMCs were stained with CD3-APC, CD4-PerCP, CD25-FITC, and CD127-PE antibodies for 15 min on ice to analyze the frequency of CD4+CD25+IL7R~c~ cells. CD4+PerCP, CD25-PE, and Alexa Fluor 488 mouse anti-human CD282 (TLR2), CD284 (TLR4), and isotype-matched control antibodies were used for adjustment of the fluorescence intensity.

Construction of plasmids. The HBV plasmids was constructed as described elsewhere, with minor modifications [29]. In brief, a serum sample from one of the consecutive patients with fulminant hepatitis B (fulminant hepatitis clone 2), whose serum level of HBV DNA was the highest of the 5 patients, was used to extract total DNA (QiAamp DNA blood mini kit; Qiagen), which was subjected to nested PCR for 2 overlapping fragments; the amplified fragments were nucleotides 1051–3215/1–1953 (fragment A) and nucleotides 180–1953 (fragment B). Then the vectors were digested with XbaI, and the XbaI-XbaI site of fragment A-pUC118 was ligated to the XbaI-XbaI site of fragment B-pUC118. Finally, a plasmid containing a 1.3-fold HBV genome (nucleotides 1051–3215/1–1953) was constructed and named pBFH2.

Cell culture and transfection. Human hepatoma HepG2 cells were incubated in Dulbecco modified Eagle medium supplemented with 10% bovine serum at 37°C and 5% carbon dioxide. For the assay of HBV replication, 6-well plates were seeded with 5 × 10^4 HepG2 or Huh7 cells each. On the next day, 1.5 μg of plasmid DNA were transfected to these cells by use of TransIT LT-1 transfection reagent (Mirus), and the culture supernatant and cells were collected 3 d later. The transfection efficiency was evaluated with a Great EscAPe secreted alkaline phosphatase reporter system 3 (Clontech), in which 10 ng/mL of a reporter plasmid expressing secreted alkaline phosphatase was cotransfected. Experiments were performed at least in triplicate.

Quantification of extracellular HBV DNA, HBsAg, and HBeAg levels. To digest the input plasmid DNA in the culture supernatant, 5 μL of the supernatant was treated with 5 U of DNase I (TaKaRa Bio) at 37°C for 1 h, and the reaction was stopped with edetic acid. Then total DNA was extracted with a QIAamp DNA blood mini kit, and 10 μL of 200-μL DNA solution was subjected to real-time PCR by use of a LightCycler
Figure 1. Effects of heat shock protein 60 (HSP60) on HBcAg-specific interleukin 10 (IL-10)–secreting regulatory T (T(\(\text{reg}\))) cells. A, Flow chart of the methods. B, Representative dot plots of IL-10-secreting cells in the CD4\(^+\)CD25\(^+\) cells. The mixed cells (antigen-presenting cells [APCs; CD4\(^+\)] and isolated CD4\(^+\)CD25\(^+\) cells) were stained with anti-CD4–peridinin chlorophyll protein complex (PerCP), anti-CD25–fluorescein isothiocyanate (FITC), and anti-IL-10–phycoerythrin (PE). The numbers in each top right quadrant indicate the frequencies of CD25\(^+\) IL-10-secreting cells among the CD4\(^+\) cells. The numbers in each bottom right quadrant indicate the frequencies of CD4\(^+\)CD25\(^+\)IL-10 cells among the CD4\(^+\) cells. The numbers in each box in the top right quadrant indicate the frequencies of CD25\(^+\) IL-10-secreting cells among the CD4\(^+\) cells. C, Representative results for a sample from 1 patient with chronic hepatitis B (samples were obtained from 3 patients with chronic hepatitis B; this experiment was repeated 3 times). Bars indicate the percentage of IL-10-secreting cells among the CD4\(^+\) cells with various kinds of pretreatment. D, Percentage of high–IL-10-secreting cells among the CD4\(^+\) cells. Error bars indicate the standard deviation of 3 independent experiments with a sample from 1 patient with chronic hepatitis B. Three independent experiments yielded similar results to those shown in panels C and D. *\(P<.05\).
system (Roche). ELISA kits were used to assay HBsAg (Hope Laboratories) and HBeAg (BioChain Institute) in 50 μL of the culture supernatant.

Sequence analysis of HBV DNA. The presence of HBV DNA in the serum samples was determined by means of PCR, as described elsewhere [30]. Nucleic acids were extracted from 100 mL of serum and subjected to nested PCR for the S gene. The amplification product of the first-round PCR was 461 bp, and that of the second-round PCR was 437 bp. The amplification products were sequenced directly on both strands by use of the BigDye Terminator Cycle Sequencing Ready reaction kit (Applied Biosystems). The presence of HBV DNA in patients with chronic hepatitis B, HBeAg-positive patients with chronic hepatitis B (HBeAg-positive and HBeAg-negative) were statistically significantly higher than those in patients with chronic hepatitis C (Figure 3). On the other hand, the mean (± SD) serum level of sHSP70 was 8.09 ± 3.64 ng/mL in patients with chronic hepatitis C, and 3.54 ± 0.46 ng/mL in healthy subjects. There were no statistically significant differences in the level of sHSP70 between the chronic hepatitis B and chronic hepatitis C patient groups (Figure 3). Then we examined the correlations between the HSP60, HSP70, and HBV DNA or ALT levels. The levels of sHSP60 were correlated with the HBV DNA levels (r = 0.532; P < .001) but not with the ALT levels (r = 0.101; P = .315) (Figures 6A and 6B). On the other hand, the levels of sHSP70 were correlated with the ALT levels (r = 0.520; P < .001) but not with the HBV DNA levels (r = 0.076; P < .449) (Figure 6C and 6D).

HBV replication could directly induce sHSP60 production in vitro. Two kinds of plasmids carrying a 1.3-fold HBV genome that could replicate in HepG2 cells were used to analyze whether HBV replication could affect the production of sHSP60 in culture medium. The transfection efficiency was almost the
Figure 4. Direct effect of hepatitis B virus (HBV) on the production of heat shock protein 60 (HSP60) and heat shock protein 70 (HSP70). Two kinds of plasmid (pBAH2 and pBFH2) carrying a 1.3-fold HBV genome that could replicate in HepG2 cells and a mock plasmid were used to analyze whether HBV replication affects the production of soluble HSP60 (sHSP60) in culture medium. The levels of sHSP60 and soluble HSP70 (sHSP70) were compared among the 3 plasmid groups. Bars indicate the levels of HSP60 (A) and HSP70 (B). The HBsAg and HBV DNA levels and standard deviations (SDs) are included below the bar graphs. C, Levels of sHSP60 in cells with and those in cells without lamivudine treatment. The cells were treated with lamivudine (Lam; 0.5 μmol/L) for 72 h. Three independent experiments were performed.
Figure 5. Suppression assay of regulatory T (T_{reg}) cells. The suppressive activity of T_{reg} cells was analyzed by means of coincubation of unstained isolated T_{reg} cells and autologous CD4^+CD25^-/H11002 cells with carboxyfluorescein succinimidyl ester (CFSE) staining. A, Representative histogram of CFSE-stained CD4^+CD25^-/H11002 effector cells and unstained CD4^+CD25^+ T_{reg} cells. B, Various levels of cell division in CD4^+CD25^-/H11002 effector cells observed 3 d after coincubation with CD3CD28-coated beads. C, Mean fluorescence intensity (MFI) of CFSE staining of CD4^+CD25^-/H11002 cells before treatment, 3 months after the start of entecavir (ETV) treatment, and 6 months after the start of entecavir treatment. The bars show the MFI of the samples divided by the MFI of the pretreatment samples \times 100. The error bars indicate the standard deviations of the data.

The effect of HSP60 on the HBCAg-specific IL-10-secreting T_{reg} cells. Previously, we found that HBCAg-specific IL-10-secreting cells could play an important role in the hyporesponsiveness of T cells in patients with chronic hepatitis B [9]. The effects of HSP60 on HBCag-specific IL-10-secreting T_{reg} cells were analyzed. The appropriate dose of rHSP60 pretreatment was determined by use of PBMCs from healthy subjects (Figure 2). Pretreatment with rHSP60 could increase the frequency of HBCag-specific IL-10-secreting cells statistically significantly (P<.01) and enhance the function of IL-10 secretion of HBCag-specific T_{reg} cells, because the frequencies of high-intensity cells with IL-10 staining in HSP60 pretreatment T_{reg} cells were statistically significantly higher than those of control groups (Figure 1D). Moreover, these effects were completely blocked by neutralizing TLR2 antibody but not by TLR4 antibody. These data indicate that HSP60 might enhance the susceptibility and function of IL-10 secretion of HBCag-specific T_{reg} cells.

Sequential analysis of clinical samples collected during entecavir therapy. Ten patients were selected for sequential analysis during entecavir therapy. The titers of HBV DNA and the ALT level rapidly decreased during entecavir therapy (Figures 7A and 7B). The serum levels of HSP60 had statistically significantly decreased at 3 months and at 6 months after the start of entecavir therapy. The frequency of T_{reg} cells and the expression level of TLR2 during entecavir treatment were quantified sequentially for up to 6 months during treatment by means of flow cytometry analysis. The frequency of CD4^+CD25^- cells decreased, although not statistically significantly. On the other hand, the frequency of CD4^-CD25^+IL7R^+ cells (subpopulation of CD4^-CD25^- cells) had statistically significantly decreased at 3 months and at 6 months after the start of entecavir therapy. The reason for the discrepancy could be that CD4^-CD25^- cells included not only T_{reg} cells but also activated CD4^+ effector cells. Previously, some research groups had found that CD4^-CD25^-FoxP3^- cells are almost the same as CD4^-CD25^-IL7R^- cells. Therefore, our data indicate that entecavir therapy could reduce the frequency of T_{reg} cells. We also investigated the frequency of CD4^-CD25^-FoxP3^+ cells during lamivudine therapy (Figure 8). The frequency of sHSP70 in the supernatant of the pBAH2- and pBFH2-transfected HepG2 cells were comparable with that of the mock-transfected HepG2 cells (Figure 4B). The addition of HBV-derived antigen in the culture supernatant could not increase the level of sHSP60 (data not shown). We performed the experiment on the suppression of HBV replication by nucleoside analogues in vitro. The suppression of HBV replication could statistically significantly reduce the production of sHSP60 (Figure 4C). These data indicate that HBV replication could increase the level of sHSP60 in the supernatant of the hepatocyte culture.
Figure 6. Analysis of the correlations between levels of heat shock proteins (HSPs), hepatitis B virus (HBV) DNA, and alanine aminotransferase (ALT). Open symbols indicate the values in samples from HBeAg-negative, HBeAb-positive patients. Filled symbols indicate the values in samples from HBeAg-positive, HBeAb-negative patients. The statistical analysis was performed by use of nonparametric Kendall \( \tau_b \) methods. An approximately straight line is included in each graph. A, Correlation between heat shock protein 60 (HSP60) level and HBV DNA level. B, Correlation between HSP60 level and ALT level. C, Correlation between heat shock protein 70 (HSP70) level and HBV DNA level. D, Correlation between HSP70 level and ALT level.
Figure 7. Sequential analysis of primary lymphocytes and soluble heat shock protein 60 (sHSP60) during entecavir (ETV) therapy. A, Representative dot plots of the CD4^+CD25^+IL7R^−/CD127 cells before treatment and 6 months after the start of treatment. Peripheral blood mononuclear cells were stained with anti-CD3, anti-CD4, anti-CD25, and anti-IL7R (CD127). The phenotypes of the CD4^+ cells were determined as follows: CD4^+CD25^+IL7R^− cells were identified as regulatory cells and CD4^+CD25^+IL7R^+ cells were identified as activated CD4^+ cells. B, Representative histogram of Toll-like receptor 2 (TLR2) surface expression on CD4^+CD25^+ regulatory T (T_{reg}) cells before treatment and 6 months after the start of treatment. C and D, Serum levels of alanine aminotransferase (ALT) and hepatitis B virus (HBV) DNA during ETV treatment. Solid black lines and error bars indicate the mean values and standard deviations, respectively. E–G, Frequencies of CD4^+CD25^+IL7R^− cells, CD4^+CD25^+ cells, and CD4^+CD25^+IL7R^+ cells among CD4^+ cells during ETV treatment, respectively. H and I, Mean fluorescence intensity (MFI) of TLR2 and Toll-like receptor 4 (TLR4) expression on CD4^+CD25^+ cells during ETV treatment. J, Serum levels of sHSP60 during ETV treatment. *P < .01 for comparison between pretreatment levels and posttreatment levels.
CD4+CD25+FoxP3+ cells was also decreased during lamivudine therapy. Moreover, the expression level of TLR2 on CD4+CD25+ cells gradually declined during entecavir therapy (Figure 7G).

Suppressive activity of Treg cells. The suppressive activity of Treg cells was analyzed by means of coinoculation of unstained isolated Treg cells and autologous CD4+CD25+ cells with CFSE staining. Ex vivo peripheral blood samples from 10 selected patients were analyzed before treatment, 3 months after the start of treatment, and 6 months after the start of treatment. The mean fluorescence intensity of the CFSE staining of the CD4+CD25+ cells was statistically significantly decreased at 6 months after the start of treatment (P<.05). These data indicate that the suppressive activity of Treg cells was gradually decreased during entecavir treatment.

DISCUSSION

In this study, we have demonstrated that the levels of sHSP60 in patients with chronic hepatitis B were statistically significantly higher than those in patients with chronic hepatitis C. Moreover, the levels of sHSP60 were correlated with the HBV DNA levels but not with the ALT levels. On the other hand, the levels of sHSP70 were correlated with the ALT levels but not with the HBV DNA levels. This discrepancy in the correlation might be due to differences in the mechanism of heat shock protein production or secretion. The release of such heat shock proteins from cells is triggered by physical trauma and behavioral stress as well as by exposure to immunological danger signals [31, 32]. Stress protein release occurs both through physiological secretion mechanisms and during cell death by necrosis [33, 34]. HSP60 might be induced by the stress of HBV replication, because the levels of HSP60 were clearly correlated with the HBV DNA levels. On the other hand, HSP70 secretion might also be caused by cell death, because the levels of sHSP70 were correlated with the ALT levels. However, we should wait for more detailed studies about the HBV-specific induction of HSP60 to confirm this correlation. Extracellular stress proteins of the heat shock protein and glucose-regulated stress protein families, including HSP60, have powerful effects on the immune response [35]. Moreover, various kinds of immune cells such as macrophages, dendritic cells, CD4+ effectector T cells, and Treg cells are affected by heat shock proteins [28, 35]. Most recently, Cohen-Sfady et al [36] reported that HSP60 enhanced the activity of IL-10 secretion from B cells. This effect could support our findings of the immune-suppressive effect of HSP60. However, we can not draw conclusions about the whole effects of immune responses because the various kinds of immune cells might affect each other by means of cytokines, chemokines, stress-related proteins, and direct binding.

In this study, we focused on the effect of HSP60 on Treg cell function by isolating Treg cells, because many research groups had reported that the function and frequency of Treg cells might be related to HBV replication. Treg cells play an important role in the immune-hyporesponsiveness of patients with chronic hepatitis B. Previously, we demonstrated that the polarization of CD4+ T cells was suppressed when the cells were stimulated with HBCAg in patients with chronic hepatitis B. Treg cells are important cells in the suppression of the T helper 1 cell response by HBCAg, as demonstrated by the increased population of IL-10-secreting CD4+CD25+ cells. This indicates the presence of an inducible Treg cell population, which is specific for HBCAg and produces IL-10, as well as a natural Treg cell population in patients with chronic hepatitis B. Pretreatment with rHSP60 increased the frequency of HBCAg-specific IL-10-secreting CD4+CD25+ cells and enhanced the IL-10-secreting activity. These results indicate that pretreatment with rHSP60 might enhance the susceptibility of the HBCAg response and the function of IL-10 production by Treg cells. These data might not imply that there was an expansion of HBCAg-specific Treg cells as a result of the rHSP60 pretreatment, because the incubation phase was for only 16 h (4 h of pretreatment with rHSP60 plus 12 h of coincubation with HBCAg-presenting APCs). However, there is a possibility that continuous exposure to sHSP60 might induce an expansion of Treg cells by enhancing the sensitivity of the expansion signal.

In this study, we found that the effect of HSP60 could be blocked by TLR2 neutralizing antibody but not by TLR4 neutralizing antibody. These data indicate that the effect of HSP60 could depend on TLR2. During entecavir therapy, not only the frequency of Treg cells but also the serum levels of HSP60 and surface expression of TLR2 on Treg cells gradually decreased. Therefore, we performed the suppression assay to detect the activity of Treg cells by use of ex vivo isolated Treg cells. The results of this suppression assay indicate that the reduction of the HBV DNA level could suppress the excessive activity of Treg cells. In our previous study, the frequency and the function of HBV-specific cytotoxic T lymphocytes were partially recovered after therapy with nucleoside or nucleotide analogues [11]. The results clearly indicate that this restoration might be due to not only the reduction of HBV antigens but also the reduction of the frequency and function of Treg cells.

On the basis of genomic analysis, 8 genotypes (A–H) of HBV have been defined, among which genotypes A, B, and especially C are prevalent in Japan [37–40]. Previous studies suggested that the clinical outcome of chronic hepatitis B was more severe in patients infected with genotype C, compared with those infected with genotype B [38, 39]. In this study, most of the
samples had HBV genotype C because of the high frequency of HBV genotype C infection in Japan. However, the expression levels of HSP60 were different among samples with the various genotypes in preliminary in vitro studies (data not shown). In addition, the expression patterns of chemokines in HBV-replicating Huh7 cells are apparently different among the various genotypes (Y. Kondo et al, unpublished data, May 2009). However, during entecavir treatment, the level of sHSP60 production in patients with genotype Bj HBV infection was quite similar to that in patients with genotype C HBV infection. We could not determine the relevance of the HBV genotypes and sHSP60 production levels because of the small numbers of genotype Bj–infected patients in this study.

In conclusion, we found that HSP60 was produced by HBV-replicating hepatocytes and determined the relevance of sHSP60 to Treg cells functions, especially for IL-10-secreting activity. The understanding of the immunopathogenesis of chronic hepatitis B could contribute to the development of novel kinds of immune therapy. Combination therapy with nucleoside or nucleotide analogues should be a reasonable method, because the suppression of HBV replication could reduce the excessive immune tolerance induced by Treg cells.

References
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