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Short Communication

Suppression of Furin by Interferon-γ and the Impact on Hepatitis B Virus Antigen Biosynthesis in Human Hepatocytes

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The roles of furin and intrahepatic cytokines in chronic heptatitis B virus (HBV) infection remain largely unknown. Here, we examined the relations between furin, IL-10, IL-12β, interferon (IFN)-γ, programed death (PD)-1, programed death ligand (PD-L)1, and the suppression of hepatitis B e antigen (HBeAg) and surface antigen (HBsAg) biosynthesis. Liver biopsies were performed on 20 chronically HBV-infected (15 HBeAg-positive and 5 HBeAg-negative) patients to assess liver inflammation/fibrosis, and mRNA levels of furin, IL-10, IL-12β, IFN-γ, PD-1, and PD-L1 were assessed by quantitative real-time PCR. IFN-γ mRNA abundance was associated with lower furin mRNA levels and higher PD-1 and PD-L1 mRNA levels in liver tissue from HBeAg-positive patients. IL-10 and IL-12β mRNA levels positively correlated with IFN-γ expression levels ($P < 0.05$). PD-L1 and furin mRNA levels were further assessed in IFN-γ-stimulated hepatoma cell lines with (HepG2.2.15 cells) and without (HepG2 and Huh7 cells) HBV replication. IFN-γ enhanced PD-L1 expression in hepatoma cells. In HepG2.2.15 cells, IFN-γ further suppressed furin and HBeAg expression. Furin inhibition and knockdown in HepG2.2.15 cells also down-regulated HBeAg and HBsAg biosynthesis. These data suggest that IFN-γ modulates the inflammatory response to avoid excessive hepatocyte damage through the enhancement of PD-1/PD-L1 expression, whereas furin suppression may contribute to a reduction in HBeAg/HBsAg biosynthesis. ***(Am J Pathol 2012, 181: 19–25; http://dx.doi.org/10.1016/j.ajpath.2012.03.036)***

During the course of chronic heptatitis B virus (HBV) infection, hepatitis B e antigen (HBeAg) seroconversion is associated with reduced viral replication and a substantial decrease in the risk of liver cirrhosis and hepatocarcinogenesis.[1–4] Most spontaneous HBeAg seroconversion occurs after puberty in chronic HBV-infected patients, and prevalence increases with age.[5,6] However, the contributing factors and mechanisms of HBeAg seroconversion and the avoidance of fulminant hepatic failure are incompletely understood.[7–9]

Our previous study found that IL-10 and IL-12β are associated with spontaneous HBeAg seroconversion at an earlier age.[10] IL-12 is a proinflammatory cytokine that modulates the immune system to act against various viral infections; IL-10 is a cytokine essential for immune regulation.[11] In animal models and cell lines, the relevant downstream signals of IL-10 (ie, programed death 1, PD-1, and its ligand, PD-L1) and IL-12 [ie, interferon (IFN)-γ] play key roles in the course of chronic HBV infection.[12–15] Furin, a proprotein peptidase located at the endoplasmic reticulum membrane in human hepatocytes, is used by HBV to facilitate the biosynthesis and maturation of HBeAg from 25-kDa proprotein to 17-kDa mature HBeAg. Furin gene polymorphism was found to affect the processing of HBeAg in human hepatocytes and the onset age of spontaneous HBeAg seroconversion in humans.[16] However, the roles of furin and intrahepatic cytokines in chronic HBV-infected persons remain largely unknown.

In this study, we assessed the relations among furin, intrahepatic cytokines (IL-10, IL-12, IFN-γ), and related

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signals (PD-1, PD-L1) in the liver parenchyma of chronic HBV-infected patients. Further *in vitro* studies that used hepatoma cell lines were also conducted to clarify the relations among the observed phenomena.

Materials and Methods

Assessment of the mRNA Expression Levels in Human Liver with Chronic HBV Infection

Subjects infected with HBV ($n = 20$) were recruited from the Department of Pediatrics of the National Taiwan University Hospital; five subjects were HBeAg-negative [3 with serum alanine aminotransferase (ALT) level <40 IU/mL, and 2 with ALT levels between 100 and 102 IU/mL]; the remaining 15 subjects were HBeAg positive (2 with serum ALT < 40 IU/mL, and 13 with ALT levels between 43 and 956 IU/mL). None of the patients was treated with IFN-α or nucleos(t)ide analogs. After the liver biopsy, specimens were stored in liquid nitrogen until processing. RNA was extracted with the RNeasy mini kit (Qiagen, Hilden, Germany) and reverse-transcribed with SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). Taqman-based quantitative real-time PCR was performed with an ABI 7900 (Applied Biosystems, Foster City, CA). The primers and probes used to detect IL-10, IL-12β, IFN-γ, furin, PD-1, and PD-L1 were commercially available (Applied Biosystems); TATA-binding protein was used as the endogenous control and reference.

The study protocol was approved by the Institutional Review Board of the National Taiwan University Hospital. All patients included in the study gave signed informed consent for liver tissue collection and storage.

HepG2, HepG2.2.15, and Huh7 Cell Lines

HepG2 cells (a human hepatoma cell line) were purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan), Huh7 cells (a human hepatocarcinoma cell line) and HepG2.2.15 cells (an HBV replication-competent human hepatoma cell line),[17] were gifts from Dr. Hui-Lin Wu (Hepatitis Research Center, National Taiwan University Hospital, Taipei, Taiwan). Cell culture media were purchased from Thermo Scientific (Logan, UT); fetal bovine serum was purchased from Invitrogen; human recombinant IFN-γ was purchased from R&D Systems, Inc. (Minneapolis, MN); TRIzol was purchased from Gibco BRL (Gaithersburg, MD); all other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

IFN-γ Treatment

HepG2.2.15 and HepG2 cells were maintained in 10% fetal bovine serum and α-modified Eagle's medium (αMEM; Thermo Scientific); Huh7 cells were cultured in 10% fetal bovine serum in DMEM (Dulbecco's modified Eagle's medium; Invitrogen); cells were subcultured every 3 days to avoid full confluence. Then, 6×10^4 cells were plated in six-well culture plates overnight in DMEM supplemented with 10% heat-inactivated fetal bovine serum, and IFN-γ (0, 15, 30 ng/mL) was added. Each culture condition was performed in triplicate. After 24 hours of incubation, HepG2 and Huh7 cells were washed with PBS (Biological Industries, Kibbutz Beit Haemek, Israel) twice and harvested for RNA extraction. HepG2.2.15 cells were washed twice with PBS and harvested for RNA extraction after 24, 48, and 72 hours of incubation. The culture medium of HepG2.2.15 cells was replaced at 24 and 48 hours. HepG2.2.15 culture medium was harvested at 24, 48, and 72 hours to quantify HBeAg secretion amount during 0 to 24, 24 to 48, and 48 to 72 hours of culture.

Gene Expression Assay

RNA extracted from HepG2, HepG2.2.15, and Huh7 cell lines was reverse-transcribed with SuperScript III Reverse Transcriptase for quantitative real-time PCR analysis. The primers and probes used were commercially available; TATA-binding protein was the endogenous control. PD-L1 and furin mRNA levels were assessed after 24 hours of IFN-γ treatment in HepG2, HepG2.2.15, and Huh7 cell lines. Levels of furin and PD-L1 mRNA were also determined in HepG2.2.15 cells after 48 and 72 hours of IFN-γ treatment.

Furin Inhibition and Knockdown in HepG2.2.15 Cells

HepG2.2.15 cells (1×10^5 cells plated in six-well culture plates) in αMEM (Thermo Scientific) supplemented with 10% heat-inactivated fetal bovine serum with and without furin inhibitor I (10 μmol/L; Decanoyl-Arg-Val-Lys-Arg-CMK; EMD Chemicals, Inc., Gibbstown, NJ) cultured *in vitro* for 11 days were used to assess the effect of furin inhibition on HBeAg and HBsAg production. Each culture condition was performed in triplicate. Furin knockdown by RNA interference was also used to assess the effect of furin on biosynthesis of HBV viral particles in HepG2.2.15 cells in αMEM with 10% heat-inactivated fetal bovine serum for 1 day. The following three small interfering RNAs (siRNAs) specific to furin (OriGene Technologies, Rockville, MD) were used: siRNA-A, SR303336A, 5′-GGACAUGAGAUAAUGUUAGAGGUdTdT-3′; siRNA-B, SR303336B, 5′-GCACUAUAGCACCGAGAAUGACGGdTdG-3′; and siRNA-C: SR303336C, 5′-CGAGGUGACAGAUGCAGUGGAGGCA-3′. Transfection was performed with TurboFect TM (Fermentas Inc., Hanover, MD), according to the manufacturer's instructions. The efficacy of furin knockdown by siRNA was further assessed by Western blot analysis with the use of standard methods.

HBeAg and HBsAg Determination in HepG2.2.15 Culture Medium

Culture media of HepG2.2.15 with and without IFN-γ treatment were obtained from 0 to 24, 24 to 48, and 48 to 72 hours of *in vitro* culture. Culture media of HepG2.2.15

Table 1. Profiles of Cytokines and Related Molecules in Liver Parenchyma in Chronic HBV-Infected Patients in HBeAg-Positive versus HBeAg-Negative Status

	HBeAg-positive (n = 15)	HBeAg-negative (n = 5)	P
Sex (M:F)	9:6	3:2	0.98
Liver biopsy age, median (range) (years)	15.6 (2.3–24.8)	14.0 (3.6–21.5)	0.90
ALT, median (range) (IU/L)	210 (16–956)	41 (11–124)	0.04
HBV genotype (B:C)	14:1	5:0	0.99
mRNA levels, median (range) ($2^{-\Delta\Delta Ct}$)			
IL-10	0.79 (0–1.10)	0.45 (0–0.94)	0.17
IL-12β	1.33 (0.94–1.72)	0.96 (0.25–1.67)	0.30
IFN-γ	0.95 (0.09–4.45)	0.43 (0.16–1.44)	0.09
Furin	0.80 (0.11–2.58)	0.61 (0.56–1.69)	0.57
PD-1	1.43 (0.25–2.80)	0.58 (0.36–1.32)	0.03
PD-L1	1.04 (0.72–1.37)	0.76 (0.36–1.16)	0.20

F, female; M, male; ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; IFN-γ, interferon-γ; PD-1, program death-1; PD-L1, program death-ligand 1.

with and without furin inhibitor treatment were collected after 1 to 5 days of *in vitro* culture. HBeAg amount in sample-to-cutoff ratio was assessed by radioimmunoassay (Abbott Laboratories, North Chicago, IL) and HBsAg titer by Architect HBsAg QT (Abbott Diagnostic, Wiesbaden, Germany).

Electron Microscopic Assessment of HBV Viral Particles in the Cytoplasm of HepG2.2.15 Cells

To determine the effect of furin inhibition on HBV viral particles in HepG2.2.15 cells, electron microscopic assessment was used to assess the structure of the HBV nucleocapsid in HepG2.2.15 cells with and without furin inhibitor I (10 μmol/L) pretreatment for 48 hours.

Enzyme-Linked Immunosorbent Assay Determination of Serum IL-10, IL12, and IFN-γ Levels

Serum samples obtained from patients at the time of liver biopsy were assessed for IL-10, IL-12, and IFN-γ levels by enzyme-linked immunosorbent assay (DuoSet; R&D Systems).

Statistical Analyses

Statistical analyses were carried out using STATA 8.2 (StataCorp LP, College Station, TX) and MedCalc 12.2.1 (MedCalc Software, Mariakerke, Belgium) software packages. The $2^{-\Delta\Delta Ct}$ values measured by quantitative real-time PCR, which represented the relative mRNA expression level, were applied for statistical analysis. The Mann-Whitney U-test was used to analyze differences in distribution/median between groups of continuous variables, and Fisher's exact test was applied to categorical variables. Simple and multiple regression analyses were used to analyze the relations between variables. Analysis of variance was used to analyze the differences in median/range between two groups of continuous variables. A P value < 0.05 was deemed to indicate statistical significance.

Results

Relation among Furin, Intrahepatic Cytokines Expression Patterns, and Clinical Data in Human Liver Specimens

Liver specimens from 15 HBeAg-positive subjects and 5 HBeAg-negative subjects were analyzed by quantitative real-time PCR (Table 1). The median age at liver biopsy of the 15 HBeAg-positive patients (nine males, six females) was 15.6 years (range, 2.3 to 24.8 years), and HBeAg seroconversion occurred at a median interval of 2.0 years (range, 0.2 to 16.7 years) after the biopsy in these patients. The median age at liver biopsy of the five HBeAg-negative patients (three males, two females) was 14.0 years (range, 3.6 to 21.5 years) at a median of 2.1 years (range; 0.9 to 2.8 years) after HBeAg seroconversion. Most of the HBeAg-positive patients had elevated serum ALT levels (>40 IU/mL) at the time of liver biopsy (86.7%), whereas 40% of the HBeAg-negative patients had elevated serum ALT levels. The mRNA levels of PD-1 were significantly higher in the livers of HBeAg-positive subjects than in the livers of HBeAg-negative subjects (Table 1). mRNA levels of IL-10, IL-12β, IFN-γ, and furin were higher in the HBeAg-positive group, but this was not statistically significant, probably because of the relatively small sample size.

In the liver specimens from HBeAg-positive subjects, IFN-γ mRNA abundance was significantly correlated with IL-10 and IL-12β expression levels [correlation coefficient (CC) = 0.67 and 0.61; P = 0.006 and 0.02, respectively; Figure 1, A and C]. Serum IL-10 and IL-12 levels at the time of liver biopsy were also positively associated with serum IFN-γ levels (CC = 0.98 and 0.99; $P < 0.001$ and $P < 0.01$, respectively). IFN-γ abundance was also associated with PD-L1 (CC = 0.84; $P < 0.001$; Figure 1B) and PD-1 (CC = 0.72; P = 0.003; Figure 1E) expression levels, PD-L1 and PD-1 mRNA levels were significantly correlated (CC = 0.61; P = 0.004), and increased IFN-γ mRNA abundance was associated with decreased furin mRNA levels (CC = −0.52; P = 0.04; Figure 1D) in patients' liver tissues. The multiple-regression model also indicated a significant association between furin and

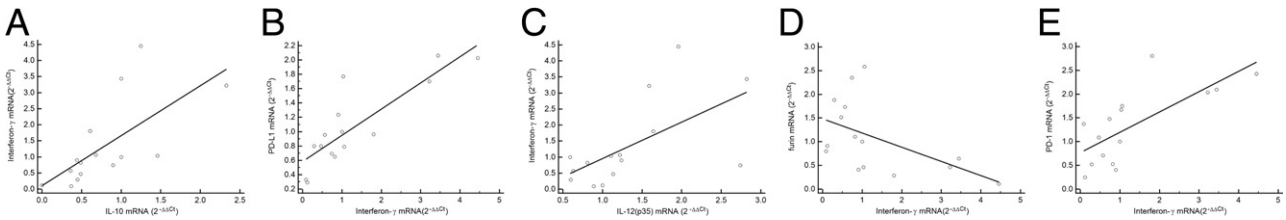

Figure 1. Correlations of mRNA levels between IL-10 and interferon (INF)-γ (**A**), IFN-γ and programed death ligand (PD-L)1 (**B**), IL-12β(p35) and INF-γ, (**C**), INF-γ and furin (**D**), and INF-γ and programed death (PD)-1 (**E**) [correlation coefficient (CC) = 0.67, 0.84, 0.61, −0.52, and 0.72; $P = 0.006$, $P < 0.001$, $P = 0.02$, $P = 0.04$, and $P = 0.003$, respectively) of liver specimens from heptatitis B e antigen-positive chronic heptatitis B surface antigen carriers.

IFN-γ mRNA levels ($r = -0.81$; $P = 0.02$) when considering the effects of IL-10 and IL-12β in the statistical model. In the multiple linear regression analysis for prediction of PD-1/PD-L1 levels, IFN-γ was the most significant predicting factor ($P < 0.01$). Higher IL-10 mRNA levels were predictive of a shorter interval from the time of liver biopsy to spontaneous HBeAg seroconversion (CC = −0.52; $P = 0.04$). In liver tissue from the HBeAg-negative subjects, there was no association of IL-10 and IL-12β with IFN-γ mRNA levels ($P > 0.05$). There were positive correlations of PD-L1 and PD-1 with IFN-γ mRNA levels ($P = 0.02$ and 0.02, respectively), and PD-1 mRNA abundance was correlated with PD-L1 mRNA level (CC = 0.92; $P = 0.03$). Furin mRNA level was also negatively correlated with IFN-γ expression ($P = 0.04$) in the livers of HBeAg-negative subjects.

Subjects with a serum ALT level fivefold or more higher than the upper normal limit ($n = 5$) at liver biopsy were noted to have significantly higher IFN-γ and PD-L1 mRNA levels ($P = 0.02$ and 0.01, respectively) than others ($n = 15$). Slightly greater IL-10, IL-12β, and PD-1 mRNA expressions in the liver of the high-ALT group ($n = 5$) compared with the others ($n = 15$) ($P = 0.08$, 0.07, and 0.09, respectively) were also noted.

Effect of IFN-γ on HBV-(HepG2.2.15) and Non-HBV-Producing Hepatoma Cell Lines (HepG2 and Huh7) in Vitro

To confirm the relations between furin and PD-L1 and IFN-γ observed in human liver tissues, we cultured non-HBV-producing hepatoma cell lines (HepG2 and Huh7) and a HBV viral particle production-competent hepatoma cell line (HepG2.2.15) *in vitro* with recombinant human IFN-γ at different concentrations (0, 15, 30 ng/mL) for 24 to 72 hours. There was a dose-dependent increase in PD-L1 expression levels in IFN-γ-stimulated HepG2 and Huh7 cell lines (Figure 2). In IFN-γ (15, 30 ng/mL; 24 hours) stimulated HepG2 and Huh7 cells, a trend toward a correlation between increased IFN-γ levels and decreased furin expression (HepG2: CC = −0.82, $P = 0.07$; Huh7: CC = −0.84, $P = 0.08$) was also observed.

The HBeAg secretion ability of HepG2.2.15 cells *in vitro* significantly decreased after IFN-γ treatment (Figure 3A). IFN-γ also enhanced PD-L1 mRNA expression in a dose-dependent manner (from 24 to 72 hours; Figure 3B). A decline in furin mRNA expression in HepG2.2.15 cells was observed after 48 and 72 hours of incubation with recombinant IFN-γ *in vitro* (Figure 3C).

Effect of Furin Suppression and Knockdown on HBV-Producing HepG2.2.15 Cells in Vitro

We further analyzed the effect of furin on IFN-γ-mediated HBV suppression with the use of a furin inhibitor-I and RNA-interference knockdown system in HepG2.2.15 cells *in vitro*. Furin inhibitor-I significantly suppressed HBeAg production by HepG2.2.15 cells, beginning on the third day of stimulation (Figure 4A). After 2 days of *in vitro* culture, the HBV nucleocapsids in the cytoplasm of HepG2.2.15 cells increased in size compared with those without furin inhibition (53.0 ± 3.6 vs 44.7 ± 2.9 nm; $P < 0.001$). HBsAg titer in the culture medium of HepG2.2.15 cells declined after the fifth day of furin inhibition (Figure 4C). Western blot analysis and quantification showed that furin production in HepG2.2.15 cells significantly decreased after knockdown by RNA interference (data not shown). Knockdown of furin in HepG2.2.15 cells by siRNA also suppressed the biosynthesis of both HBeAg and HBsAg (Figure 4, B and D).

Discussion

Clinical data describing intrahepatic cytokine downstream signal expression levels in chronic HBV-infected patients before and after HBeAg seroconversion are limited but valuable in elucidating the HBeAg seroconversion process.

Furin is a proprotein convertase in hepatocyte located at the membrane of the endoplasmic reticulum.[18] Furin is essential for the maturation and secretion of HBeAg from hepatocytes by cleaving the C-terminus of the HBV pre-core proprotein (P22) and maintaining peripheral immune

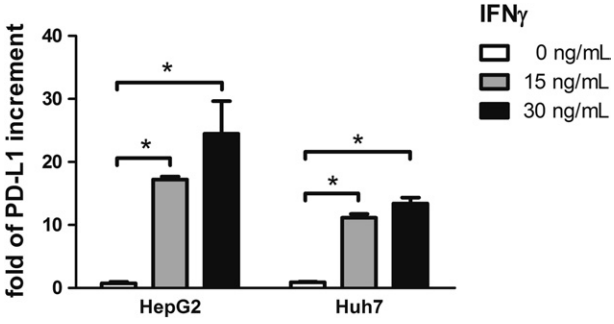

Figure 2. Programed death ligand (PD-L)1 mRNA expression in HepG2 and Huh7 cancer cell lines after interferon (IFN)-γ stimulation (0, 15, or 30 ng/mL). PD-L1 mRNA level was calculated as the ratio of the $2^{-\Delta\Delta Ct}$ values with and without IFN-γ treatment. *$P < 0.001$.

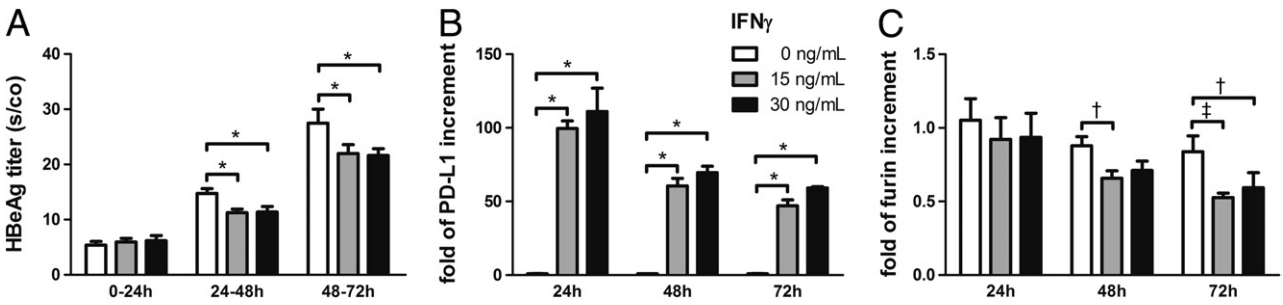

Figure 3. mRNA levels of heptatitis B virus e antigen (HBeAg) (**A**), programed death ligand (PD-L)1 (**B**), and furin (**C**) in HepG2.2.15 cell-culture medium after interferon (IFN-γ) *in vitro* stimulation. Expression in HepG2.2.15 cells after IFN-γ stimulation (0, 15, or 30 ng/mL). $^{*}P < 0.001$. S/CO, sample-to-cutoff ratio, $^{\dagger}P < 0.05$, and $^{\ddagger}P < 0.01$.

tolerance.[18–20] Failure to process the HBV P22 into mature HBeAg (P17) disrupts the stability of HBV nucleocapsids and down-regulates HBV replication via false integration of the HBV P22 into the HBV nucleocapsid (P21) polymer.[21,22]

In this study, we identified a negative correlation between IFN-γ and furin expression in the livers of chronically HBV-infected patients before HBeAg seroconversion. Furin inhibition was also found by electron microscopy to alter the structure of HBV nucleocapsids in the cytoplasm of HepG2.2.15 cells, beginning on the third day of furin inhibition, followed by a decline in HBsAg titer in culture medium after the fifth day of culture. This change in HBV nucleocapsid structure suggests that the nonsecreted HBeAg P22 may have some effect on the HBV nucleocapsid P21 polymer architecture and that the altered structure further inhibits HBsAg biosynthesis. The HBeAg P22 proprotein was noted to misincorporate into the HBV nucleocapsid polymer in a previous study and was suspected to associate with the similar amino acid sequence and structure of the P21 and P22 proteins.[22] Our results showed poor HBeAg secretion after furin inhibition, which may lead to the misincorporation of HBeAg P22 proprotein into the HBV nucleocapsid polymer, alter the structure of the HBV nucleocapsid, and decrease the efficacy of HBV replication and HBsAg biosynthesis. These observations suggest a new regulatory role for IFN-γ during the course of spontaneous HBeAg seroconversion by the down-regulation of furin expression, which may decrease the secretion of both HBeAg and HBsAg.[20–22]

In this study, we also observed positive correlations between IL-10 and IFN-γ and between IL-12 and IFN-γ levels in liver tissues and serum of patients before HBeAg seroconversion. Subjects with more severe hepatitis activity indicated by higher ALT levels were also noted to have elevated intrahepatic IFN-γ, PD-1/PD-L1, IL-10, IL-12β, and PD-1 mRNA expressions. None of these subjects with high ALT levels developed hepatic failure after the ALT peak. IFN-γ, an important antiviral cytokine, was reported to suppress HBV replication, HBV nucleocapsid assembly, and even pregenomic RNA synthesis in a noncytolytic manner.[23] IL-12, a proinflammatory cytokine, promotes type I helper T-cell differentiation and suppresses type 2 helper T-cell function. It also enhances the production of antiviral cytokines, such as INF-γ and IL-2, by mononuclear cells and amplifies the cytotoxicity of cytotoxic T lymphocyte (CTL) and natural killer cells.[24] IL-12 is associated with cytolytic destruction of HBV-infected hepatocytes by CTLs and noncytolytic antiviral effects mediated by downstream IFN-γ.[25] Most chronic HBV-infected patients may experience hepatitis flare-up at the inflammatory phase, followed by an inactive phase after HBeAg seroconversion, with a decline in viral load and normalization of liver-function profile. However, the transition from cytolytic to noncytolytic HBV suppression to avoid excessive liver damage and hepatic failure are not well understood. IL-10 is considered to be an immunomodulator during viral infections and was also noted to enhance the secretion of INF-γ by CTLs in the presence of IL-2, a downstream signal of IL-12.[26,27] Higher intrahepatic IL-10 mRNA levels were associated with a shorter interval from the time of liver biopsy to the occurrence of spontaneous HBeAg seroconversion in the present study and are compatible with our previous observation in serum.[10]

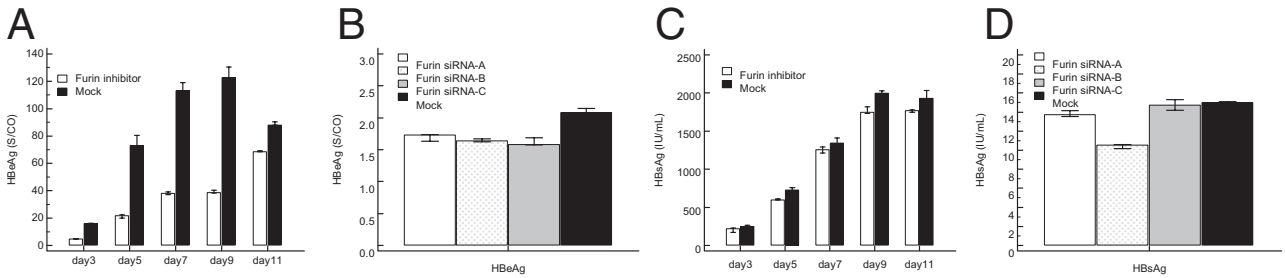

Figure 4. Effect of furin inhibitor-I on the secretion of heptatitis B virus e antigen (HBeAg) (**A**) and heptatitis B virus surface antigen (HBsAg) (**C**) by HepG2.2.15 cells. Effect of furin knockdown on HBeAg (**B**) and HBsAg (**D**) biosynthesis in HepG2.2.15 cells. S/CO, sample-to-cutoff ratio; siRNA, small interfering RNA.

We also observed positive correlation of IFN-γ with PD-1 and PD-L1 mRNA levels in HBeAg-positive human livers. PD-1 is an immune-regulatory receptor belonging to the CD28/CTLA-4 family. It negatively regulates antigen receptor signals on interaction with either PD-L1 or PD-L2.[28] We restricted our investigations to PD-L1 because this is the ligand of PD-1 expressed on hepatocytes. In HepG2, HepG2.2.15, and Huh7 hepatoma cell lines, we demonstrated the IFN-γ dose-dependent induction of PD-L1 mRNA expression. PD-1/PD-L1 is considered a marker of immunologic tolerance and T-cell dysfunction in the presence of infectious pathogens, including HBV.[14,29] Blockage of PD-1/PD-L1 was found to enhance the re-activation of HBV-specific CTLs and secretion of IFN-γ by circulating intrahepatic lymphocytes after HBeAg seroconversion.[30] Strikingly, nonexpression of the PD-1/PD-L1 pathway was associated with fulminant hepatic failure in HBV-infected patients.[31] Hence, up-regulation of the PD-1/PD-L1 pathway may efficiently mitigate pathogenic T-cell responses, limit liver damage, and avoid massive hepatocyte damage and fulminate hepatic failure in patients with HBV infection.[31] PD-1/PD-L1, noted to be induced in the liver by viral infection and IFN-γ, is thus suspected to play key regulatory roles in avoiding excessive tissue damage once the inflammatory response is programed to turn off or the immune response fails to clear the pathogen.[32,33] In this study, increased intrahepatic IFN-γ mRNA levels in HBeAg-positive chronic HBV-infected patients were correlated with elevated PD-1/PD-L1 mRNA abundances, and recombinant IFN-γ enhanced PD-L1 expression in hepatoma cells. An increase in PD-L1 expression in the liver during the inflammatory phase may represent an oscillation in the immune response, whereby a cessation of the excessive inflammatory response follows active inflammation to avoid excessive liver damage.[31–33]

In conclusion, this is the first study to report decreased HBV viral antigen biosynthesis (HBeAg and HBsAg) by IFN-γ-mediated furin down-regulation, whereas PD-1/PD-L1 pathway up-regulation may modulate the inflammatory response to avoid excessive liver damage and fulminant hepatic failure. These findings offer some new insight into antiviral therapy of chronically HBV-infected patients.

Acknowledgments

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References

1. Law YF, Lau GK, Kao JH, Gane E: Hepatitis B e antigen seroconversion: a critical event in chronic hepatitis B virus infection. Dig Dis Sci 2010, 55:2727–2734
2. Liu Z, Luo K, He H, Hou J: Hot-spot mutations in hepatitis B virus core gene: eliciting or evading immune clearance? J Viral Hepat 2005, 12:146–153
3. Nordenfelt E, Andren-Sandberg M: Dane particle-associated DNA polymerase and e antigen: relation to chronic hepatitis among carriers of hepatitis B surface antigen. J Infect Dis 1976, 134:85–89
4. Fattovich G, Rugge M, Brollo L, Pontisso P, Noventa F, Guido M, Alberti A, Realdi G: Clinical, virologic, and histologic outcome following seroconversion from HBeAg to anti-HBe in chronic hepatitis type B. Hepatology 1986, 6:167–172
5. Wu JF, Tsai WY, Hsu HY, Ni YH, Chen HL, Tsuei DJ, Chang MH: Effect of puberty onset on spontaneous hepatitis B virus e antigen seroconversion in men. Gastroenterology 2010, 138:942–948
6. Chang MH, Sung JL, Lee CY, Chen CJ, Chen JS, Hsu HY, Lee PI, Chen DS: Factors affecting clearance of hepatitis B e antigen in hepatitis B surface antigen carrier children. J Pediatr 1989, 115:385–390
7. Hsu HY, Chang MH, Hsieh KH, Lee CY, Lin HH, Hwang LH, Chen PJ, Chen DS: Cellular immune response to HBcAg in mother-to-infant transmission of hepatitis B virus. Hepatology 1992, 15:770–776
8. Rosenberg W: Mechanisms of immune escape in viral hepatitis. Gut 1999, 44:759–764
9. Bertoni R, Sidney J, Fowler P, Chesnut RW, Chisari FV, Sette A: Human histocompatibility leukocyte antigen-binding supermotifs predict broadly cross-reactive cytotoxic T lymphocyte responses in patients with acute hepatitis. J Clin Invest 1997, 100:503–513
10. Wu JF, Wu TC, Chen CH, Ni YH, Chen HL, Hsu HY, Chang MH: Serum levels of interleukin 10 and 12 predict early, spontaneous hepatitis B virus e antigen seroconversion. Gastroenterology 2010, 138:165–172
11. Mocellin S, Panelli MC, Wang E, Nagorsen D, Marincola FM: The dual role of IL-10. Trends Immunol 2003, 24:36–43
12. Guidotti LG, McClary H, Loudis JM, Chisari FV: Nitric oxide inhibits hepatitis B virus replication in the liver of transgenic mice. J Exp Med 2000, 191:1247–1252
13. Amaro MJ, Bartolome J, Carreno V: Hepatitis B virus X protein transactivates the inducible nitric oxide synthase promoter. Hepatology 1999, 29:915–923
14. Evans A, Riva A, Cooksley H, Phillips S, Puranik S, Nathwani A, Brett S, Chokshi S, Naoumov NV: Programmed death 1 expression during antiviral treatment of chronic hepatitis B: impact of hepatitis B e-antigen seroconversion. Hepatology 2008, 48:759–769
15. McNaughton L, Puttagunta L, Martinez-Cuesta MA, Kneteman N, Mayers I, Moqbel R, Hamid Q, Radomski MW: Distribution of nitric oxide synthase in normal and cirrhotic human liver. Proc Natl Acad Sci U S A 2002, 99:17161–17166
16. Lei RX, Shi H, Peng XM, Zhu YH, Cheng J, Chen GH: Influence of a single nucleotide polymorphism in the P1 promoter of the furin gene on transcription activity and hepatitis B virus infection. Hepatology 2009, 50:763–771
17. Deres K, Schröder CH, Paessens A, Goldmann S, Hacker HJ, Weber O, Krämer T, Niewöhner U, Pleiss U, Stoltefuss J, Graef E, Koletzki D, Masantschek RN, Reimann A, Jaeger R, Gross R, Beckermann B, Schlemmer KH, Haebich D, Rübsamen-Waigmann H: Inhibition of hepatitis B virus replication by drug-induced depletion of nucleocapsids. Science 2003, 299:893–896
18. Messageot F, Salhi S, Eon P, Rossignol JM: Proteolytic processing of the hepatitis B virus e antigen precursor. J Biol Chem 2003, 10:891–895
19. Ito K, Kim KH, Lok ASF, Tong S: Characterization of genotype-specific carboxyl-terminal cleavage sites of hepatitis B virus e antigen precursor and identification of furin as the candidate enzyme. J Virol 2009, 83:3507–3517
20. Pesu M, Watford WT, Wei L, Xu L, Fuss I, Strober W, Andersson J, Shevach EM, Quezado M, Bouladoux N, Roebroek A, Belkaid Y, Creemers J, O'Shea JJ: T-cell-expressed proprotein convertase furin is essential for maintenance of peripheral immune tolerance. Nature 2008, 455:246–250
21. Scaglioni PP, Melegari M, Wands JR: Posttranscriptional regulation of hepatitis B virus replication by the precore protein. J Virol 1997, 71:345–353
22. Kock J, Nassal M, Deres K, Blum HE, von Weizsacker F: Hepatitis B virus nucleocapsids formed by carboxy-terminally mutated core proteins contained spliced viral genomes but lack full-size DNA. J Virol 2004, 78:13812–13818
23. Zhang Z, Zhang S, Zou Z, Shi J, Zhao J, Fan R, Qin E, Li B, Li Z, Xu X, Fu J, Zhang J, Gao B, Tian Z, Wang FS: Hypercytolytic activity of hepatic natural killer cells correlates with liver injury in chronic hepatitis B patients. Hepatology 2011, 53:73–85
24. Trinchieri G: Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu Rev Immunol 1995, 13:251–276

25. Rigopoulou EI, Suri D, Chokshi S, Mullerova I, Rice S, Tedder RS, Williams R, Naoumov NV: Lamivudine plus interleukin-12 combination therapy in chronic hepatitis B, antiviral and immunological activity. Hepatology 2005, 42:1028–1036
26. Chen WF, Zlotnik A: IL-10: a novel cytotoxic T cell differentiation factor. J Immunol 1991, 147:528–534
27. Santin AD, Hermonat PL, Ravaggi A, Bellone S, Pecorelli S, Roman JJ, Parham GP, Cannon MJ: Interleukin-10 increases Th1 cytokine production and cytotoxic potential in human papilloma-virus-specific CD8+ cytotoxic T lymphocytes. J Virol 2000, 74:4729–4737
28. Okazaki T, Honj T: PD-1 and PD-1 ligands: from discovery to clinical application. Int Immunol 2007, 1:1–12
29. Peng G, Li S, Wu W, Tan X, Chen Y, Chen Z: PD-1 upregulation is associated with HBV-specific T cell dysfunction in chronic hepatitis B patients. Mol Immunol 2008, 45:963–970
30. Fisicaro P, Valdatta C, Massari M, Loggi E, Biasini E, Sacchelli L, Cavallo MC, Silini EM, Andreone P, Missale G, Ferrari C: Antiviral intrahepatic T-cell responses can be restored by blocking programmed death-1 pathway in chronic hepatitis B. Gastroenterology 2010, 138:682–693
31. Zhang Z, Zhang JY, Wherry EJ, Jin B, Xu B, Zou ZS, Zhang SY, Li BS, Wang HF, Wu H, Lau GK, Fu YX, Wang FS: Dynamic programmed death 1 expression by virus-specific CD8 T cells correlates with the outcome of acute hepatitis B. Gastroenterology 2008, 134:1938–1949
32. Isogawa M, Furuichi Y, Chisari FV: Oscillating CD8(+) T cell effector functions after antigen recognition in the liver. Immunity 2005, 23:53–56
33. Mühlbauer M, Fleck M, Schütz C, Weiss T, Froh M, Blank C, Schölmerich J, Hellerbrand C: PD-L1 is induced in hepatocytes by viral infection and by interferon-alpha and -gamma and mediates T cell apoptosis. J Hepatol 2006, 45:520–528