study, IFN therapy was not related to HBsAg seroclearance. This difference might be attributable to the difference in the HBV genotype, the small number of patients with IFN treatment, or the past use of IFN.

The average number of platelets in the patients with HBsAg seroclearance did not change after HBsAg seroclearance. In contrast, three of eight patients with less than 150 000/μl of platelets showed an increase in platelets, which was also reported in a previous study [21]. We have reported that the number of platelets is one of the most important factors predicting the prognosis of HBV carriers [22,23]. We do not know the reason for the difference between those with and without an increase in platelets after HBsAg seroclearance; therefore, we should clarify this in the future.

In conclusion, the predictive factor for the seroclearance of HBsAg was a lower level of HBsAg. Therefore, measurement of HBsAg level is one of the most effective means to follow up HBV carriers accurately.

Acknowledgements

Conflicts of Interest

The authors thank our staffs for their help. We have no conflict of interest disclosure.

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IVYSPRING INTERNATIONAL PUBLISHER

International Journal of Medical Sciences

2012; 9(1):27-32

Research Paper

Efficacy of Lamivudine or Entecavir on Acute Exacerbation of Chronic Hepatitis B

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Received: 2011.09.21; Accepted: 2011.10.31; Published: 2011.11.10

Abstract

Background/Aims: Spontaneous acute exacerbation of chronic hepatitis B virus (HBV) infection occasionally occurs in its natural history, sometimes leading rapidly to fatal hepatic failure. We compared the effects of lamivudine (LAM) with those of entecavir (ETV) treatments in acute exacerbation of chronic hepatitis B with 500 IU/L or higher alanine aminotransferase (ALT) levels.

Methods: Thirty-four patients with acute exacerbation were consecutively treated with LAM /ETV. Their clinical improvements were compared.

Results: Among LAM-treated and ETV-treated patients, none showed a reduction of <1 log IU/mL in HBV DNA after 1 or 3 months of treatment. Initial virological response, defined as a reduction of 4 log IU/mL in HBV DNA at 6 months, with LAM and ETV, respectively, was 83.3% and 100%. One LAM patient developed hepatic encephalopathy, but all patients in both groups survived. Twelve months after treatment, 41.6% of 24 LAM group patients switched to another drug or added adefovir to their treatment due to the emergence of LAM-resistant mutants. On the other hand, patients receiving ETV did not need to change drugs.

Conclusions: ETV appears to be as effective as LAM in the treatment of patients with acute exacerbation of chronic hepatitis B. Clinicians should carefully start to treat these patients as soon as possible.

Key words: acute exacerbation, ALT, entecavir, HBV, lamivudine

INTRODUCTION

Chronic hepatitis B infection is associated with the development of hepatocellular carcinoma [1]. Infection with hepatitis B virus (HBV) also leads to wide a spectrum of liver injury, including acute, self-limited infection, fulminant hepatitis, and chronic hepatitis with progression to cirrhosis and liver failure, as well as to an asymptomatic chronic carrier state [2, 3].

Reactivation of hepatitis B is a well-characterized syndrome marked by the abrupt reappearance or rise of HBV DNA in the serum of a patient with previously inactivated or resolved HBV infection [4]. Reac-

tivation is often spontaneous, but can also be triggered by cancer chemotherapy and immune suppression. Spontaneous acute exacerbation of chronic hepatitis B infection is seen with a cumulative probability of 15-37% after 4 years of follow-up [5]. Prognosis is generally poor in HBV carriers with spontaneous acute exacerbation together with high alanine aminotransferase (ALT) levels, jaundice, and liver failure [4, 6, 7]. This condition has been defined as acute-on-chronic liver failure according to a recent Asia-Pacific consensus recommendation [8]. Acute exacerbation occasionally leads to a critical scenario, meaning that clinicians need to treat this condition immediately.

Lamivudine (LAM) is a reverse-transcriptase inhibitor of viral DNA polymerase with an excellent profile of safety and tolerability, causing inhibition of viral replication, and it is approved for antiviral treatment of hepatitis B patients [9, 10]. LAM suppresses serum HBV DNA values in up to 98% of patients within a median period of 4 weeks, leading to aminotransferase normalization, increased hepatitis B e antigen (HBeAg) seroconversion rate, and improvement of histological parameters [11, 12]. A study from Taiwan showed that LAM had a survival benefit and was effective for patients with baseline bilirubin levels below 20 mg/dL [7].

Entecavir (ETV), a deoxyguanosine analogue, is a potent and selective inhibitor of HBV replication; its *in vitro* potency is 100- to 1,000-fold greater than that of LAM, and it has a selectivity index (concentration of drug reducing the viable cell number by 50% [CC_{50}]/concentration of drug reducing viral replication by 50% [EC_{50}]) of ~8,000 [13, 14]. At present, the Japanese national health insurance system approves ETV as the first-line therapy for chronic hepatitis B, although some patients are treated with standard interferon-alfa. ETV is a nucleoside analogue (NUC) belonging to a new subgroup, cyclopentane [15], and it has been shown to be highly effective in suppressing HBV replication to an undetectable level and normalizing ALT, although NUCs do not eradicate the virus. ETV develops less resistance than LAM.

We undertook a retrospective study to compare the efficacy of LAM with that of ETV in the reduction of HBV DNA levels and associated improvement in disease severity and biochemical recovery in patients with acute exacerbation together with higher ALT levels due to HBV reactivation.

MATERIALS AND METHODS

Patients

A retrospective analysis of LAM/ETV-treated chronic hepatitis B patients at Chiba University Hospital and Numazu City Hospital, Japan, between May 2003 and December 2009 was performed. The inclusion criteria were: acute exacerbation of chronic hepatitis B characterized by an elevation of ALT level ≥ 500 IU/L along with HBV DNA ≥ 4.5 log IU/mL presenting in a patient with diagnosed chronic liver disease. The exclusion criteria were: acute hepatitis B, superinfection with other viruses (hepatitis E, A, D, or C), other causes of chronic liver failure [16, 17], coexistent hepatocellular carcinoma, portal thrombosis, coexistent renal impairment, pregnancy, coinfection with human immunodeficiency virus (HIV), or patients who had received a previous course of NUC treatment. This retrospective study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by the Ethics Committee of Chiba University, Graduate School of Medicine [18].

Baseline assessment of patients

Retrospectively collected data included patient demographics, clinical findings, all laboratory variables including virological tests and abdominal ultrasound. HBsAg, HBeAg, anti-HBe antibody and immunoglobulin M (IgM) anti-HBc antibody were determined by ELISA (Abbott, Chicago, IL, USA) or CLEIA (Fujirebio, Tokyo, Japan) [19]. HBV genotype was determined from patients' sera by ELISA (Institute of Immunology, Tokyo, Japan) as reported by Usuda et al [20]. HBV DNA was measured by Roche Amplicor™ PCR assay (detection limits: 2.6 log IU/mL; Roche Diagnostics, Tokyo, Japan).

Definitions

Primary antiviral treatment failure was defined as a reduction of < 1 log IU/mL in HBV DNA after 3 months of therapy. Initial virological response (IVR) was defined as a reduction of ≥ 4 log IU/mL in HBV DNA after 6 months of therapy [21].

Follow-up

Clinical assessment and routine investigations were done every 15 days or every month for at least 6 months. HBV DNA measurements were repeated monthly.

Statistical analysis

Statistical analyses were performed using Microsoft Excel 2010 for Windows™ 7 and StatView 5 (SAS Institute Inc, Cary, NC). Continuous variables were expressed as mean ± standard deviation and were compared by two-factor analysis of variance (ANOVA) and two-way repeated measures ANOVA. Categorical variables were compared by Chi-square

test. Baseline was taken as the date when the first dose of LAM/ETV was administered. Statistical significance was considered at a *P*-value < 0.05.

RESULTS

Patients

Between May 2003 and December 2009, 34 patients with spontaneous acute exacerbation of chronic hepatitis B, with ALT levels ≥ 500 IU/mL and treated with LAM or ETV, were consecutively enrolled and retrospectively analyzed. 24 (70.5%) were treated with LAM at 100 mg daily and 10 (29.4%) were treated with ETV at 0.5 mg daily. All patients were followed for at least 6 months. Mean follow-up in the LAM and ETV groups was 55.5 ± 25.4 and 16.5 ± 9.9 months, respectively.

Baseline characteristics

Baseline characteristics in the two patient groups were similar **(Table 1)**. Median age was 37 (21-73) years and 79.4% were men. One patient of the LAM group developed hepatic encephalopathy, but recovered. All patients in both groups survived. At admission, the serological profile showed HBsAg positivity in all 34 (100%); 22 (64.7%) were HBeAg positive. The median HBV DNA level was 7.4 log IU/mL in the LAM group and 7.9 log IU/mL in the ETV group **(Table 1)**.

Table 1 Demographic, Clinical, and Laboratory Variables of Patients at Entry.

Parameters	Total Patients (N=34)	LAM (N=24)	ETV (N=10)	*P*-value
Age (years)	37 (21-73)	37 (21-73)	39 (24-67)	NS
Male (%)	27 (79.4)	18 (75)	9 (90)	NS
Cirrhosis (+/-)	2/32	2/22	0/10	NS
ALT (IU/L)	986 (523-2,450)	995 (523-2,450)	1,046 (523-2,140)	NS
T. Bil (mg/dL)	2.0 (0.8-22.0)	2.4 (0.8-20.6)	1.6 (1.9-22.0)	NS
PT (%)	83 (24-121)	81.5 (24-119)	83.6 (35-121)	NS
HBeAg (+/-)	22/12	18/6	4/6	NS
HBV DNA (log IU/mL)	7.6 (4.8-8.7)	7.4 (5.2-8.7)	7.9 (4.8-8.7)	NS

LAM, lamivudine; ETV, entecavir; ALT, alanine aminotransferase; T. BIL, total bilirubin; PT, prothrombin time; NS, statistically not significant.

Reduction in HBV DNA of total patients

LAM significantly reduced HBV DNA levels from baseline 7.24 log IU/mL to 3.27 log IU/mL at 1 month ($P < 0.001$), to 2.21 log IU/mL at 3 months ($P < 0.001$), and to 1.53 log IU/mL at 6 months ($P < 0.001$). ETV also significantly reduced HBV DNA levels from baseline 7.56 log IU/mL to 3.12 log IU/mL at 1 month ($P < 0.001$), to 2.14 log IU/mL at 3 months ($P < 0.001$), and to 1.77 log IU/mL at 6 months ($P < 0.001$). There were no differences in HBV DNA levels from baseline to 6 months between the two groups. None with primary antiviral treatment failure was identified in either group. There were no significant differences in IVR between the two groups **(Figure 1)**.

Reduction in ALT levels of total patients

LAM significantly reduced ALT levels from baseline 1,130 IU/mL to 102 ($P < 0.001$) at 1 month, to 28.6 ($P < 0.001$) at 3 months, and to 23.1 ($P < 0.001$) at 6 months. ETV also significantly reduced ALT levels from baseline 1,210 IU/mL to 117 ($P < 0.001$) at 1 month, to 25 ($P < 0.001$) at 3 months, and to 24.4 ($P < 0.001$) at 6 months. There were no differences in ALT levels from baseline to 6 months between the two groups **(Figure 2)**.

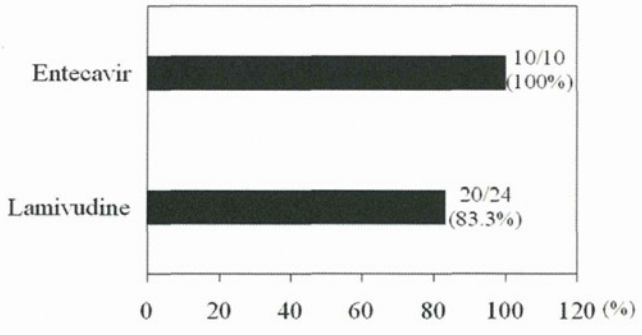

Figure 1 Initial virological response (IVR). IVR was defined as a reduction of ≥ 4 log IU/mL in HBV DNA after 6 months of therapy [21].

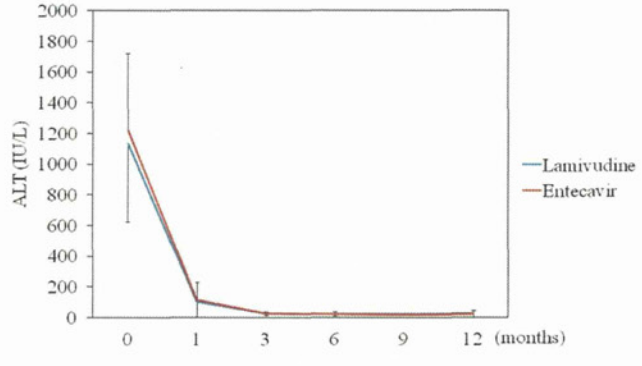

Figure 2 Efficacy of lamivudine and entecavir for ALT levels. Lamivudine (N=24) vs. entecavir (N=10); data are shown as mean ± SD.

Reduction in HBV DNA of HBeAg-positive patients

It has been demonstrated that the levels of HBV DNA in the HBeAg-positive phase were generally higher than those in the ant-HBe-positive phase [19, 22]. HBeAg positivity is also associated with HBV viremia and increased ALT levels in HIV/HBV co-infected patients [23]. Next, we compared the response to LAM or ETV in 18 or 4 HBeAg-positive patients, respectively **(Table 2)**. LAM significantly reduced HBV DNA levels from baseline 7.52 log IU/mL to 3.35 log IU/mL ($P < 0.001$) at 1 month, to 2.38 log IU/mL ($P < 0.001$) at 3 months, and to 1.55 log IU/mL ($P < 0.001$) at 6 months. ETV also significantly reduced HBV DNA levels from baseline 8.42 log IU/mL to 3.87 log IU/mL ($P < 0.001$) at 1 month, to 2.90 log IU/mL ($P < 0.001$) at 3 months, and to 2.22 log IU/mL ($P < 0.001$) at 6 months. There were no differences in HBV DNA levels from baseline to 6 months between the two groups. Primary antiviral treatment failure was not observed in either group. Four patients in the LAM group did not achieve IVR.

Table 2 Demographic, Clinical, and Laboratory Variables of HBeAg-positive Patients at Entry.

Parameters	Total Patients (N=22)	LAM (N=18)	ETV (N=4)	*P*-value
Age (years)	34.5 (21-51)	36.5 (21-51)	30 (24-33)	NS
Male (%)	18 (81.8)	14 (77.7)	4 (100)	NS
Cirrhosis (+/-)	1/21	1/17	0/4	NS
ALT (IU/L)	1,030 (523-2,450)	1,990 (523-2,450)	1,363 (980-1,620)	NS
T. Bil (mg/dL)	1.75 (0.8-20.6)	2.0 (0.8-20.6)	1.5 (1.0-18.7)	NS
PT (%)	77 (24-119)	73.6 (24-119)	95.0 (44.1-113)	NS
HBeAg (+)	22	18	4	
HBV DNA (log IU/mL)	7.6 (5.5- 8.8)	7.6 (5.5- 8.7)	8.6 (7.6- 8.7)	NS

LAM, lamivudine; ETV, entecavir; ALT, alanine aminotransferase; T. BIL, total bilirubin; PT, prothrombin time; NS, statistically not significant.

Reduction in ALT levels of HBeAg-positive patients

LAM significantly reduced ALT levels from baseline 1,150 IU/mL to 84 ($P < 0.001$) at 1 month, to 27.5 ($P < 0.001$) at 3 months, and to 22.0 ($P < 0.001$) at 6 months. ETV also significantly reduced ALT levels from baseline 1,460 IU/mL to 230 ($P = 0.0038$) at 1 month, to 22.2 ($P = 0.0016$) at 3 months, and to 24.0 ($P = 0.0016$) at 6 months. At 1 month after treatment, the ALT levels of the LAM groups were lower than those of the ETV group ($P < 0.0001$) **(Figure 3)**. During follow-up periods, 10 and 1 sero-converters of HBeAg to anti-HBe antibody phase were seen in 18 LAM-treated and in 4 ETV-treated patients, respectively.

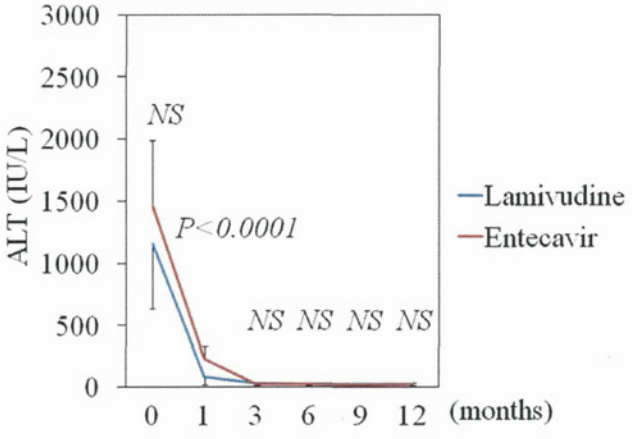

Figure 3 Efficacy of lamivudine and entecavir for ALT levels in HBeAg-positive patients. Lamivudine (N=18) vs. entecavir (N=4); data are shown as mean ± SD.

Safety

No patient stopped taking medications. Twelve months after treatment, 10 of 24 patients (41.6%) in the LAM group switched from LAM to ETV (n=4) or added adefovir (n=6) due to the emergence of LAM-resistant mutants. On the other hand, patients receiving ETV did not need to change their medication.

DISCUSSION

The present study compared the use of NUCs, LAM and ETV, for the treatment of acute exacerbation of chronic hepatitis B. The results clearly showed significant benefits of a rapid reduction of HBV DNA levels, compared with untreated patients in a previous report [4].

It was reported that ETV treatment is associated with increased short-term mortality in patients with severe acute exacerbation of chronic hepatitis B, but that it achieves better virological response in the long run [24]. We used LAM or ETV for patients with acute exacerbation of chronic hepatitis B presenting with ALT ≥ 500 IU/L in the present study. The effects of LAM on HBV DNA levels were the same as those of ETV (Figure 1). But the effects of LAM on ALT levels after 1 month were stronger than those of ETV in HBeAg-positive patients **(Figure 3)**. In spite of the limited number of these patients, the effects were possibly related to immunomodulating activities of LAM [25]. The patients' prognoses were more favorable than in the previous report [4]. This might have

depended on the fact that, in the present study, treatment was begun as soon as possible, and some patients may have had a milder grade of acute exacerbation of chronic hepatitis B than those in the previous report [4]. We believe that patients with acute exacerbation of chronic hepatitis B need to be subjected to treatment as promptly as possible.

The major routes of HBV infection in our country have been mother-to-child transmission and blood transfusion. However, cases with HBV transmitted through sexual contact are increasing, especially among HIV-1-seropositive patients [26]. One should bear in mind that knowledge about interactions between ETV and anti-HIV nucleoside analogues is limited [27]. Because long-term use of LAM induces LAM-resistant mutants [28], we can only use LAM for short-term treatment of patients with acute exacerbation of chronic hepatitis B. On the other hand, the present study also revealed that patients receiving ETV did not need to change drugs.

Recently, there have been several reports that reactivation of HBV is a fatal complication following systemic chemotherapy or other immunosuppressive therapy including rituximab and steroid therapies mainly in HBsAg-positive and -negative lymphoma patients. It is important to enable early diagnosis of HBV reactivation as well as initiation of antiviral therapy [29, 30].

In conclusion, ETV appears to be as effective as LAM in the treatment of patients with acute exacerbation of chronic hepatitis B. Clinicians should start to treat these patients with NUCs as soon as possible.

ACKNOWLEDGEMENTS

This work was supported by the Japan Science and Technology Agency, Ministry of Education, Culture, Sports, Science and Technology, Japan [21590829 (TK), 21590828 (FI), and 21390225 (OY)], a grant from the Japan Society of Hepatology (TK), and a grant from Chiba University Young Research-Oriented Faculty Member Development Program in Bioscience Areas (TK).

This work was partly presented at 21st Conference of the Asian Pacific Association for the Study of the Liver on 17 February 2011, Queen Sirkit National Convention Center, Bangkok, Thailand.

ABBREVIATIONS

ETV: Entecavir; HIV: Human immunodeficiency virus; IVR: Initial virological response; LAM: Lamivudine; NUC: Nucleoside analogue.

CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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HEPATOLOGY
Official Journal of the American Association for the Study of Liver Diseases

CORRESPONDENCE

Child-Pugh Score Is Altered by the Albumin Measurement Method

To the Editor:

Serum albumin level is one of the important measures for Child-Pugh classification score that indicates liver insufficiency.[1] Bromocresol green (BCG), which is used for conventional serum albumin measurement, reacts with proteins other than albumin. This results in the overestimation of albumin levels. The traditional bromocresol purple (BCP) method is highly specific for albumin, but reacts differently depending on the albumin form (e.g., mercaptalbumin, nonmercaptalbumin, albumin bound to bilirubin). On the other hand, the modified BCP methods are highly specific for albumin and are not affected by albumin form, thus allowing accurate and reproducible serum albumin measurement.[2] In Japan, many laboratories have substituted the modified BCP method for the BCG method, and 45% of laboratories employed the modified BCP method in 2011. However, the modified BCP method generates lower values than does the BCG method. Thus, substituting the modified BCP method for the BCG method is likely to alter a patient's Child-Pugh class.

The objectives of the present study were (1) to compare serum albumin values that were determined by the BCG method and the modified BCP method in patients with liver cirrhosis (LC) and in patients with hepatocellular carcinoma (HCC) with underlying LC, and (2) to test whether the different reagents used to determine the serum albumin levels can alter the Child-Pugh classification. The serum albumin concentrations of 103 patients with LC or HCC were determined by immunonephelometry (N-Antiserum to Human Albumin; Siemens, Tokyo, Japan), the BCG method (ALB-A; Sysmex, Tokyo, Japan), and the modified BCP method (Albumin-II HA Test Wako; Wako Pure Chemicals Industries Ltd., Osaka, Japan). Patients provided informed consent.

Serum albumin levels measured by the modified BCP method were well correlated with the levels measured by immunonephelometry (gold standard) (Fig. 1). Serum albumin levels obtained by the BCG method were significantly higher than the levels measured by the modified BCP method ($P = 0.031$, Student t test). This overestimation of the albumin level by the BCG method resulted in a lower albumin score in the Child-Pugh classification in 11 of the 103 patients. Of 14 patients with an albumin score of 2 by using the BCG method, 2 patients were re-scored as 3 by the modified BCP method. Of 66 patients with an albumin score of 1 by using the BCG method, 9 patients were re-scored as 2 by the modified BCP method. This re-scoring resulted in a change in Child-Pugh class from A to B in another patient and from B to C in another patient when the modified BCP method was employed instead of the BCG method.

Thus, new criteria should be set in institutions that employ the modified BCP method. The threshold values for the scoring in the Child-Pugh classification were 28.0 g/L and 35.0 g/L. The threshold values for the modified BCP method were calculated as 25.3 g/L and 32.9 g/L from the regression equation ($y = 1.076x - 4.8$) between the BCG (x) and modified BCP (y) methods. Institutions should examine these criteria to set new criteria for the modified method. The method by which serum albumin is measured should be specified in both clinical and research settings.

In conclusion, the modified BCP method provided more accurate albumin measurements than did the BCG method. Overestimation of serum albumin levels by the BCG method can alter both the Child-Pugh score and thereby the Child-Pugh class in patients with LC and HCC.

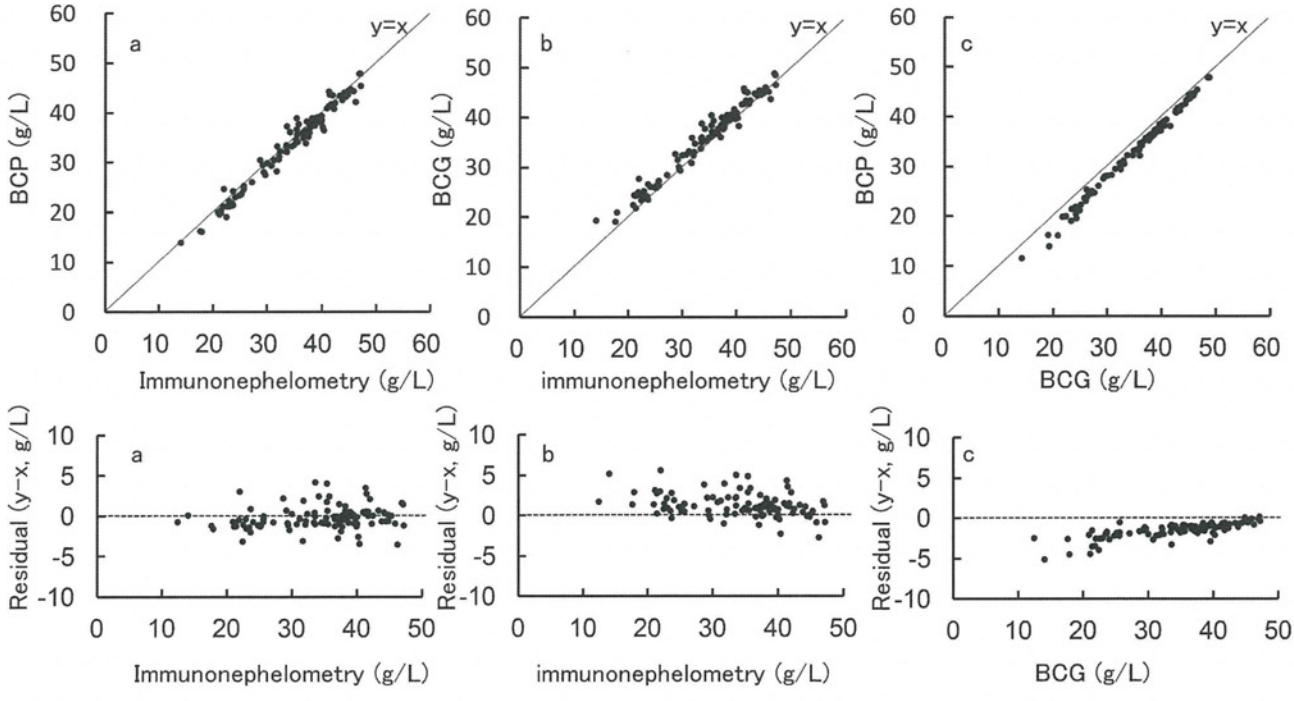

Fig. 1. Correlations of Serum albumin levels in patients between immunonephelometry and bromocresol green (BCG) and modified bromocresol purple (BCP) methods a, Correlation between immunonephelometry (x) and modified BCP method (y), r=0.991, y=1.031x−1.8; b, Correlation between immunonephelometry (x) and BCG method (y), r=0.983, y=0.954x+3.0; c, Correlation between BCG (x) and modified BCP method (y), r=0.993, y=1.076x−4.8

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DOI 10.1002/hep.25972
Potential conflict of interest: Nothing to report.

Hepatitis B Virus e Antigen Physically Associates With Receptor-Interacting Serine/Threonine Protein Kinase 2 and Regulates *IL-6* Gene Expression

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We previously reported that hepatitis B virus (HBV) e antigen (HBeAg) inhibits production of interleukin 6 by suppressing NF-κB activation. NF-κB is known to be activated through receptor-interacting serine/threonine protein kinase 2 (RIPK2), and we examined the mechanisms of interleukin 6 regulation by HBeAg. HBeAg inhibits RIPK2 expression and interacts with RIPK2, which may represent 2 mechanisms through which HBeAg blocks nucleotide-binding oligomerization domain-containing protein 1 ligand–induced NF-κB activation in HepG2 cells. Our findings identified novel molecular mechanisms whereby HBeAg modulates intracellular signaling pathways by targeting RIPK2, supporting the concept that HBeAg could impair both innate and adaptive immune responses to promote chronic HBV infection.

Hepatitis B virus (HBV) nucleoprotein exists in 2 forms [1, 2]. Nucleocapsid, designated HBV core antigen (HBcAg), is an intracellular, 21-kDa protein that self-assembles into particles that encapsidate viral genome and polymerase and is essential for function and maturation of virion. HBV also secretes a nonparticle second form of the nucleoprotein, designated precore or HBV e antigen (HBeAg) [1, 2]. Precore and core proteins are translated from 2 RNA species that have different 5′ initiation sites. Precore messenger RNA (mRNA) encodes a hydrophobic signal sequence that directs precore protein to the endoplasmic reticulum, where it undergoes N- and C-terminal cleavage within the secretory pathway and is secreted as an 18-kDa monomeric protein [3–5].

Nucleotide-binding oligomerization domain–containing protein 1 (NOD1) and NOD2 are cytosolic pattern-recognition receptors involved in the sensing of bacterial peptidoglycan subcomponents [6]. NOD1 and NOD2 stimulation activates NF-κB through receptor-interacting serine/threonine protein kinase 2 (RIPK2; also known as RIP2, RICK, or CARDIAK), a caspase-recruitment domain-containing kinase. RIPK2 is also involved in Toll-like receptor (TLR)–signaling pathway and plays an important role in the production of inflammatory cytokines through NF-κB activation [6, 7].

We previously reported that HBeAg inhibits the production of interleukin 6 (IL-6) through suppression of NF-κB activation [4]. In the present study, we investigated the molecular mechanism of HBeAg functions for the requirement of RIPK2 in NF-κB transcriptional regulation.

METHODS

Cell Culture and Plasmids

HepG2, Huh7, HT1080, COS7, and HEK293T cells were used in the present study. Stable cell lines were obtained as previously described [4]. Briefly, HepG2, Huh7, and HT1080 were transfected with pCXN2-HBeAg(+) or pCXN2-HBeAg(−) in Effectene (Qiagen). After G418 screening, HBeAg-positive and -negative HepG2/Huh7/HT1080 cell lines were collected for further analysis [4]. The plasmid pCXN2-HBeAg(+), which can produce both HBeAg and core peptides, and the plasmid pCXN2-HBeAg(−), which can produce only core peptides, were obtained as described previously [4]. pNF-κB–luc, which expresses luciferase upon promoter activation by NF-κB, was purchased from Stratagene [4]. pGFP–human RIPK2 (kindly provided by Prof John C. Reed, Sanford-Burnham Institute for Medical Research) can express GFP-human $RIP2^{WT}$ [8].

HepG2 cells were transfected with plasmid control–small hairpin RNA (shRNA) or with RIPK2-shRNA (Santa Cruz). After puromycin screening, individual colonies were picked up and examined for expression of endogenous RIPK2, and clones HepG2-shC and HepG2-shRIPK2-3 were selected for subsequent studies.

Received 1 January 2012; accepted 3 February 2012; electronically published 21 May 2012.

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The Journal of Infectious Diseases 2012;206:415–20

DOI: 10.1093/infdis/jis363

Luciferase Assays and Treatment of Cells With NOD Ligands

Around 1.0×10^5 HepG2 and Huh7 cells were plated in 6-well plates (Iwaki Glass, Tokyo, Japan) for 24 hours and transfected with 0.4 μg of pNF-κB–luc. For luciferase assay of NF-κB activation, cells were treated for 4 hours with or without NOD1 ligand (C12-iEDAP, 2.5 μg/mL) and NOD2 ligand (muramyl dipeptide [MDP], 10 μg/mL) (InvivoGen) at 44 hours after transfection [9]. After 48 hours, cells were lysed with reporter lysis buffer (Promega), and luciferase activity was determined as described previously [4].

RNA Extraction, Complementary DNA (cDNA) Synthesis, Real-Time Polymerase Chain Reaction (PCR) Analysis, and PCR Array

Total RNA was isolated by RNeasy Mini Kit (Qiagen). A total of 5 μg of RNA was reverse transcribed using the First Strand cDNA Synthesis Kit (Qiagen) [4]. Quantitative amplification of cDNA was monitored with SYBR Green by real-time PCR in a 7300 Real-Time PCR system (Applied Biosystems). Gene expression profiling of 84 TLR-related genes was performed using RT^2 profiler PCR arrays (Qiagen) in accordance with the manufacturer's instructions [4].

Gene expression was normalized to 2 internal controls (GAPDH and/or β-actin) to determine the fold-change in gene expression between the test sample (HBeAg-positive HepG2/Huh7/HT1080) and the control sample (HBeAg-negative HepG2/Huh7/HT1080) by the 2^{-ddCT} (comparative cycle threshold) method [4]. Three sets of real-time PCR arrays were performed. Some results of HepG2 cells were previously reported [4].

Coimmunoprecipitation

Cells were cotransfected with 2.5 μg pCXN2-HBeAg(+) or 2.5 μg pCXN2-HBeAg(−), as well as with 2.5 μg pGFP–human RIPK2, and cell lysates were prepared after 48 hours, using lysis buffer containing a cocktail of protease inhibitors. Cell lysates were incubated with anti-GFP rabbit polyclonal antibody (Santa Cruz) or anti-HBe mouse monoclonal antibody (Institute of Immunology, Tokyo, Japan) for 3 hours at 4°C, followed by overnight incubation with protein G–Sepharose beads (Santa Cruz). Immunoprecipitates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose membrane. Immunoblotting was performed by incubating the membrane for 1 hour with anti-HBe antibody. Proteins were detected by enhanced chemiluminescence (GE Healthcare), using an image analyzer (LAS-4000, Fuji Film). The membrane was reprobed with a monoclonal antibody to GFP or RIPK2 (Cell Signaling).

Transfection of pGFP–Human RIPK2 and Confocal Microscopy

Formaldehyde (3.7%)–fixed cells were incubated with anti-HBe antibody and stained with fluorochrome-conjugated secondary antibody (Alexa Fluor 555 conjugate, Cell Signaling). Cells were mounted for confocal microscopy (ECLIPSE TE 2000-U, Nikon). Whenever necessary, images were merged digitally to monitor colocalization. Cotransfection of 0.1 μg pCXN2-HBeAg(+) or 0.1 μg pCXN2-HBeAg(−) with 0.3 μg pGFP–human RIPK2 into the cells was performed. After 48 hours, intracellular localization of RIPK2 was visualized by confocal microscopy.

Enzyme-Linked Immunosorbent Assay (ELISA) for IL-6

Cell culture fluid was analyzed for IL-6 by ELISA (KOMA-BIOTECH, Seoul, Korea), in accordance with the manufacturer's protocol [4].

Small Interfering RNA (siRNA) Transfection and Wound-Healing Assay

Control siRNA (siC) and siRNA specific for RIPK2 (siRIPK2) were purchased from Thermo Fisher Scientific. Cells were transfected with siRNA by electroporation. After 48 hours, cells were treated with 10 ng/mL tumor necrosis factor α (TNF-α) (Wako Pure Chemical, Osaka, Japan), while the wound-healing (ie, scratch) assay was performed using a p-200 pipette tip to induce RIPK2 [10]. Up to 12 hours after scratching, the cells were observed by microscopy. Cell migration was measured using Scion Images (SAS). Migration by siC-transfected cells was set at 1.

Statistical Analysis

Results are expressed as mean values ± SD. The Student *t* test was used to determine statistical significance.

RESULTS

HBeAg Downregulates RIPK2 Expression

To explore the effect of HBeAg on TLR-related gene expression, we generated HepG2, Huh7, and HT1080 cell lines that stably expressed HBV core region with or without precore region. HT1080, a primate fibrosarcoma cell line, is useful for the study of interferon signaling. HBeAg and HBV core–related antigen (HBcrAg) levels of these cell lines demonstrated that expression of HBV core region without HBV precore region did not allow HBeAg secretion by cells (data are cited elsewhere [4] or not shown). First, we performed real-time RT-PCR analysis of these cell lines, using focused gene arrays (Figure 1*A*). We observed that, in 3 cell lines, 5 genes (*RIPK2, TLR9, TNF, CD180,* and *IL1A*) were downregulated ≥1.3-fold in HBeAg-positive cells than in HBeAg-negative cells. We chose to focus our investigation on RIPK2 because HBeAg inhibits the production of IL-6 through the suppression of NF-κB activation [4], and NF-κB is known to be activated through RIPK2 [4]. RIPK2 expression was >100-, 1.41-, and 1.45-fold lower in HBeAg-positive HepG2, Huh7, and HT1080 cells, respectively, compared with their HBeAg-negative counterparts

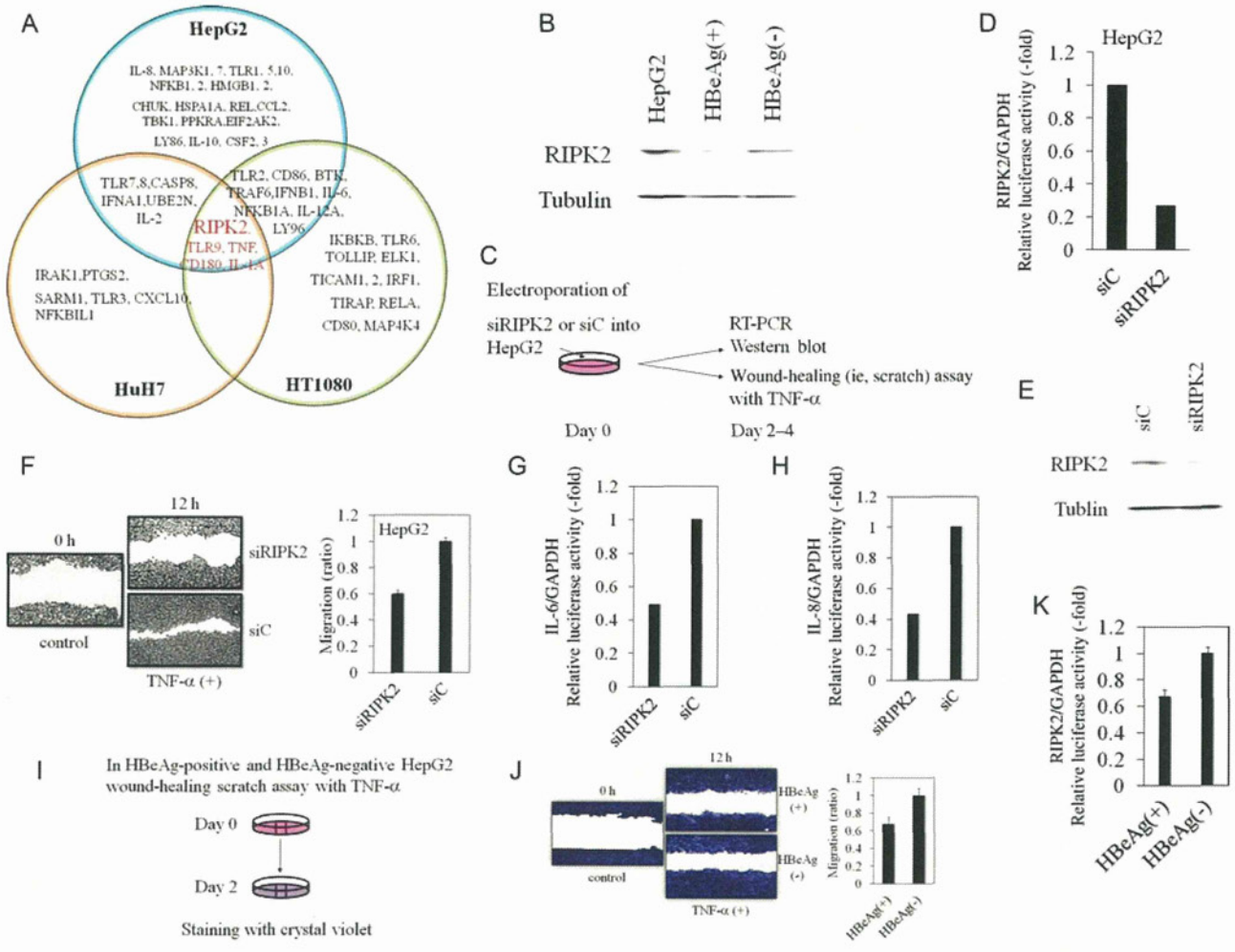

Figure 1. Receptor-interacting serine/threonine protein kinase 2 (RIPK2) expression is downregulated by hepatitis B virus e antigen (HBeAg), and knockdown of RIPK2 and HBeAg impairs hepatic wound repair. *A*, Venn diagram representing Toll-like receptor (TLR)–related genes downregulated ≥1.3-fold in HBeAg-positive HepG2/Huh7/HT1080 cells, compared with HBeAg-negative cells. Cellular RNA was extracted and analyzed with focused array, quantifying 84 genes. Gene expression levels were normalized to actin and GAPDH expression levels. *B*, HBeAg downregulates RIPK2 expression in HepG2 cells. Western blot analysis of RIPK2 and tubulin expression in HepG2, HBeAg(+) HepG2, and HBeAg(−) HepG2. *C*, Experimental protocol of electroporation of control (siC) and RIPK2 (siRIPK2) small interfering RNA (siRNA) into HepG2 cells. *D* and *E*, Real-time polymerase chain reaction (PCR; *D*) and Western blot (*E*) analyses of RIPK2 expression in siC- or siRIPK2-expressing HepG2 cells. RIPK2 messenger RNA (mRNA) levels were normalized to GAPDH levels. *F–H*, siC- and siRIPK2-transfected HepG2 cells were scratch wounded and incubated with 10 ng/mL tumor necrosis factor α (TNF-α), and cell migration was analyzed after 12 hours and quantified using Scion Image (*F*). Interleukin 6 (IL-6; *G*) and interleukin 8 (IL-8; *H*) mRNA expression are quantified by real-time reverse transcription–PCR (RT-PCR) and expressed relative to GAPDH mRNA expression. *I*, Protocol of wound-healing (ie, scratch) assay in HBeAg(+) and HBeAg(−) HepG2 cells. TNF-α was used at 10 ng/mL. *J*, Cell migration was analyzed using Scion Image. *K*, RIPK2 mRNA expression was quantified by real-time RT-PCR and expressed relative to GAPDH mRNA expression. Primers specific for RIPK2 were 5′-AGACACTACTGACATCCAAG-3′ (sense) and 5′-CACAAGTATTTCCGGGTAAG-3′ (antisense), and primers for other genes were as described previously [4]. Data are mean values ± SD of 3 independent experiments.

(Figure 1*A*). Western blot analyses confirmed lower levels of RIPK2 in HBeAg-positive HepG2 than in HBe-negative HepG2 or parental HepG2 (Figure 1*B*). The fact that RIPK2 is one of the targets for the ubiquitin proteasome system and uses a ubiquitin-dependent mechanism to achieve NF-κB activation [6] might be a reason for the differences between RIPK2 mRNA and protein expression status. We also observed lower levels of RIPK2 mRNA expression (0.18-fold) in HepG2.2.15 cells, which secrete complete HBV virion and HBeAg, compared with expression in HepG2 cells (data not shown).

Knockdown of RIPK2 and HBeAg Impairs Hepatic Cell Migration

It has recently been reported that RIPK2 expression is induced by TNF-α plus scratch wounding in keratinocytes [10]. Therefore, we next examined whether RIPK2 affected hepatic

wound healing in the presence of TNF-α in vitro (Figure 1*C*). As shown in Figure 1*D* and 1E, RIPK2 mRNA and protein expression were efficiently decreased in HepG2 cells transfected with RIPK2 siRNA (siRIPK2), but not control (siC). RIPK2 silencing reduced hepatic wound closure 1.8-fold, which was associated with a 2-fold decrease in IL-6 production, known to be an important cytokine for the regeneration of liver [11], and a 2.3-fold decrease in interleukin 8 production (Figure 1*F–H*). Importantly, RIPK2 silencing did not affect cell viability (data not shown).

Given that HBeAg downregulates RIPK2 expression (Figure 1*A* and 1*B*), we examined whether HBeAg has an effect on hepatic wound healing in the presence of TNF-α (Figure 1*I*). As expected, we observed that both cell migration

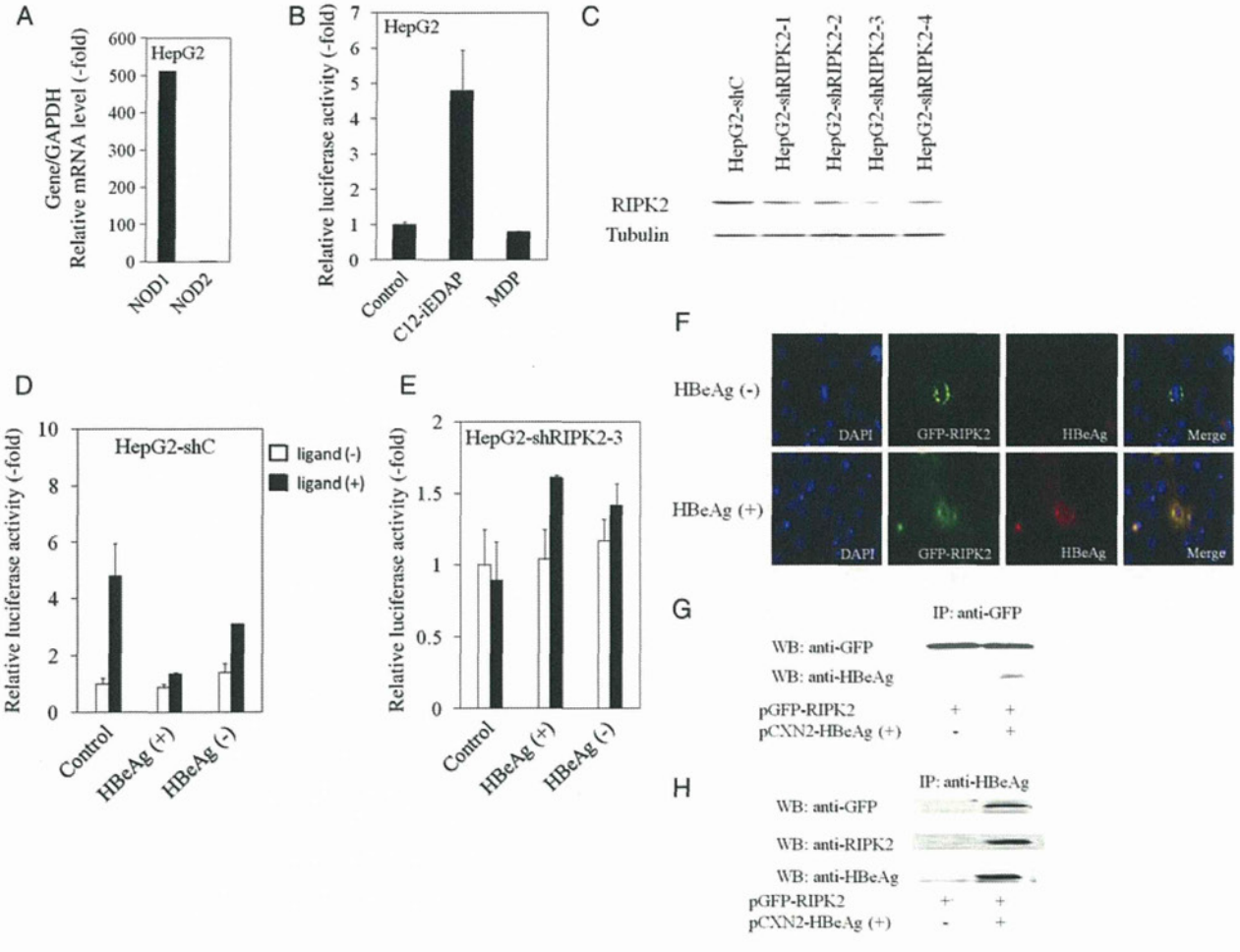

Figure 2. The nucleotide-binding oligomerization domain–containing protein 1 (NOD1) ligand C12-iEDAP induces NF-κB activation, knockdown of receptor-interacting serine/threonine protein kinase 2 (RIPK2) inhibits NOD1 ligand–induced NF-κB activation in HepG2 cells, and hepatitis B virus e antigen (HBeAg) interacts with RIPK2. *A*, Real-time reverse transcription–polymerase chain reaction analysis of NOD1 and NOD2 messenger RNA expression in HepG2. NOD1 and NOD2 expression levels were normalized to GAPDH expression levels. *B*, NF-κB–driven luciferase activity in HepG2 cells stimulated with the NOD1 ligand C12-iEDAP or the NOD2 ligand muramyl dipeptide (MDP) in HepG2. *C*, Western blot analysis of RIPK2 and tubulin expression in HepG2 cells stably transfected with control small hairpin RNA (shRNA; HepG2-shC) or with RIPK2 shRNA (HepG2–shRIPK2-1/2-4) expressing plasmids. *D* and *E*, HepG2-shC (*D*) and HepG2–shRIPK2-3 (*E*) cell lines were transiently transfected with pCXN2, pCXN2-HBeAg(+), or pCXN2-HBeAg(−) plasmids together with pNF-κB–luc. Cells were treated for 4 hours, with or without NOD1 ligand C12-iEDAP (2.5 μg/mL), and luciferase activity was determined. Primers specific for NOD1 (sense primer: 5′-ACTACCTCAAGCTGACCTAC-3′; antisense primer: 5′-CTGGTTTACGCTGAGTCTG-3′), for NOD2 (sense primer: 5′-CCTTGCATGCAGGCAGAAC-3′; antisense primer: 5′-TCTGTTGCCCCAGAATCCC-3′), and for other genes as described previously were purchased from Sigma [4]. *F*, HBeAg specifically colocalizes with RIPK2. COS7 cells were transiently cotransfected with 0.1 μg pCXN2-HBeAg(+) or pCXN2-HBeAg(−) together with 0.3 μg pGFP–human RIPK2. HBeAg was revealed with anti-HBeAg primary antibody and Alexa-Fluor-548 secondary antibody. *G* and *H*, HEK293T cells were transiently transfected with or without GFP-RIPK2 and HBeAg-expressing plasmids. Cellular extracts were precleared with protein G–Sepharose, and interacting complexes were immunoprecipitated (IP) with either anti-GFP (*G*) or anti-HBeAg (*H*) antibodies. Immunocomplexes were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and proteins were visualized by immunoblotting (WB) with indicated antibodies. Results are representative of 3 independent experiments.

and RIPK2 mRNA expression were reduced in HBeAg-positive HepG2 cells, compared with HBeAg-negative cells (1.5-fold decrease; Figure 1*J* and 1*K*). These results suggest that HBeAg impairs hepatic cell migration–dependent RIPK2 expression. Among NF-κB–targeting genes, expression of vimentin mRNA was impaired in HepG2-shRIP2 and in HBeAg-positive HepG2 (data not shown), and vimentin might be one of the candidates for impairment of their migrations [12].

RIPK2 Plays an Important Role in NF-κB Activation Induced by NOD1 Ligand, and HBeAg Blocks This Pathway

HepG2 cells express NOD1 but not NOD2 at the mRNA level (Figure 2*A*). In agreement with this finding, NF-κB was activated in HepG2 cells exposed to NOD1 ligand C12-iEDAP (level of activation, 4.8-fold, compared with untreated control) but not in those exposed to NOD2 ligand MDP (Figure 2*B*). As for Huh7 cells, activation of NF-κB was not detected following exposure to C12-iEDAP or MDP (data not shown). These results suggest that C12-iEDAP triggered NF-κB activation through NOD1 in HepG2 cells, which is consistent with findings from a previous study [9].

We examined whether knockdown of RIPK2 has an effect on NOD1-induced NF-κB activation in HepG2 cells. First, we established HepG2 cell lines that constitutively expressed RIPK2-shRNA (HepG2-shRIPK2-1/2-4) or control-shRNA (HepG2-shC) (Figure 2*C*). The HepG2–shRIPK2-3 cell line, which expresses the lowest levels of RIPK2, and the HepG2-shC cell line were treated for 4 hours, with or without C12-iE-DAP, before measurement by the NF-κB–driven luciferase assay (Figure 2*D* and 2*E*). C12-iEDAP triggered NF-κB activation in HepG2-shC (Figure 2*D*) but not in HepG2–shRIPK2-3 (Figure 2*E*), indicating that RIPK2 plays an important role in NF-κB activation induced through NOD1 triggering.

To assess the influence of HBeAg in that pathway, we measured NOD1-mediated NF-κB activity in HepG2-shC and HepG2–shRIPK2-3 cell lines transiently transfected with HBeAg-expressing plasmids. As shown in Figure 2*D*, HBeAg expression in HepG2-shC abolished C12-iEDAP–induced NF-κB activation.

HBeAg Interacts With RIPK2 and Colocalizes With RIPK2

RIPK2 mediates activation of transcription factors, such as NF-κB, following its activation, which is initiated by membrane-bound or intracytosolic receptors, such as TLR, NOD1, and NOD2 [7, 13, 14]. Confocal microscopy analysis of cells transfected with GFP-RIPK2 revealed subcellular localization of RIPK2 (data not shown). To compare the localization of RIPK2 with that of HBeAg, cells were cotransfected with pGFP–human RIPK2 with pCXN2-HBeAg(+) or pCXN2-HBeAg(−). After 48 hours, cells were stained with mouse monoclonal anti-HBe antibody. Confocal microscopy suggested subcellular colocalization of RIPK2 with HBeAg (Figure 2*F*). Reinforcing this assumption, GFP-RIPK2 coimmunoprecipitated with HBeAg (Figure 2*G*), while HBeAg coimmunoprecipitated with RIPK2 (Figure 2*H*) in transiently transfected cells with RIPK2- and HBeAg-expressing plasmids.

DISCUSSION

In the present study, we have shown the expression of NOD1 and NOD1 ligand–induced NF-κB activation in HepG2 cells and that RIPK2 plays an important role in NOD1 ligand–induced NF-κB activation. NF-κB activation plays an essential role in the production of inflammatory cytokines such as IL-6, which HBeAg could suppress in hepatocytes [4]. We have also shown that HBeAg inhibits RIPK2 expression and interacts with RIPK2, which may represent 2 mechanisms through which HBeAg blocks NOD1 ligand–induced NF-κB activation, thus contributing to the pathogenesis of chronic HBV infection and establishing viral persistence, although further studies including clinical situations might be needed.

HBeAg can be secreted by hepatocytes. Yet, it has been reported that as much as 80% of the precore protein p22 remains localized to the cytoplasm rather than undergoing further cleavage that allows its secretion as mature HBeAg [15]. Our present study showed subcellular colocalization of HBeAg with RIPK2 (Figure 2*F*). In addition to HBeAg protein in cell culture medium, we observed similar inhibition of NF-κB activation (data not shown).

Overall, we provided a novel molecular mechanism whereby HBeAg modulates innate immune signal-transduction pathways through RIPK2. Elsewhere, it was also reported that HBeAg impairs cytotoxic T-lymphocyte activity [2]. HBeAg inhibits RIPK2 expression and interacts with RIPK2, decreasing NF-κB activation and inflammatory cytokine production in hepatocytes. Taken together, HBeAg could impair both innate and adaptive immune responses to promote chronic HBV infection.

Notes

Acknowledgments. We thank Prof John C. Reed and Prof Junichi Miyazaki, for providing the plasmids, and Ms. Satomi Hasegawa, for providing technical assistance.

Financial support. This work was supported by the Japan Science and Technology Agency, Ministry of Education, Culture, Sports, Science, and Technology, Japan (21590829 to T. K. and 21590828 to F. I.); the Japan Society of Hepatology (T. K.); the Chiba University Young Research-Oriented Faculty Member Development Program in Bioscience Areas (T. K.); and the Research Grant-in-Aid from Miyakawa Memorial Research Foundation (W. S.).

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Hepatology Research

Hepatology Research 2012; 42: 1100–1111 doi: 10.1111/j.1872-034X.2012.01028.x

Original Article

Aldehyde dehydrogenase 1 is associated with recurrence-free survival but not stem cell-like properties in hepatocellular carcinoma

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Aim: It has been reported that aldehyde dehydrogenase 1 A1 (ALDH1) could be not only a normal stem cell marker but also a cancer stem cell marker. ALDH1 expression could be a predictor of poor prognosis in a wide range of cancers. However, the role of ALDH1 in hepatocellular carcinoma (HCC) remains unclear.

Method: We conducted loss-of-function assays for ALDH1 by using short-hairpin RNA in HCC cells and evaluated the correlation between ALDH1 expression and clinicopathological features based on immunohistochemical assessment of 49 primary HCC tissues.

Results: Neither cell proliferation nor the anchorage-independent sphere formation ability of HCC cells were altered after *ALDH1* knockdown. Flow cytometric analyses revealed that *ALDH1* knockdown showed no remarkable change in the proportion of epithelial cell adhesion molecule $(EpCAM)^+$ tumor-initiating cells. Although non-tumor tissues in primary HCC samples diffusely and homogenously expressed ALDH1 at low levels, tumor tissues contained cells with high levels of ALDH1 expression at varying frequencies. Primary HCC samples were categorized as ALDH1-high or ALDH1-low based on the percentage of ALDH1-overexpressing cells. ALDH1-high HCC was characterized by low serum levels of α-fetoprotein ($P < 0.01$) and well-differentiated pathology ($P = 0.03$). Multivariate analysis showed that high ALDH1 expression was a favorable prognostic factor in recurrence-free survival of HCC ($P = 0.02$).

Conclusion: Our findings show that ALDH1 expression has little association with stem cell-like features in HCC cells. ALDH1 might function as a differentiation marker rather than a stem cell marker in HCC.

Key words: aldehyde dehydrogenase 1 A1, epithelial cell adhesion molecule, hepatocellular carcinoma, cancer stem cell, tumor-initiating cell

INTRODUCTION

ALDEHYDE DEHYDROGENASE (ALDH) is a ubiquitous intracellular enzyme that catalyzes the irreversible oxidation of a variety of cellular aldehydes.[1] The enzyme shows various biological functions including cellular detoxification. The human ALDH superfamily is composed of 19 putatively functional genes including ALDH1A1 (ALDH1) and ALDH3A1 (ALDH3).[2] Of interest, it has been reported that ALDH1, a predominant isoform in mammalian tissues, serves as a stem cell marker in somatic stem cells such as hematopoietic stem cells (HSC) and neural stem cells (NSC).[3,4] In addition, ALDH1 promotes the differentiation of HSC by oxidization of retinol to retinoic acid.[5] Together, these findings indicate that ALDH1 acts as not only a stem cell marker, but also a functional molecule.

According to the recent "cancer stem cell" hypothesis, tumors contain a minor component of tumorigenic cells and a major component of non-tumorigenic cells.[6] The

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Received 23 October 2011; revision 7 April 2012; accepted 9 April 2012.

minor population, termed cancer stem cells (CSC) or tumor-initiating cells (TIC), shares the characteristics of normal stem cells such as self-renewal capability and shows pronounced tumorigenic activity in xenograft transplantation assays.[7] Recent reports implicate ALDH1 as a useful cancer stem cell marker that could be used to enrich tumor-initiating subpopulations from various cell lines and primary tumors.[8–10] Of interest, it has been documented that a high level of ALDH1 expression correlates with highly malignant phenotypes and poor clinical outcome in a range of cancers.[11,12] Taken together, it appears that ALDH1 is an important biological marker in cancers. ALDH1 and ALDH2 are highly expressed in the liver and play a crucial role in the major oxidative pathway of alcoholic metabolism.[13] However, the biological effect of ALDH1 in the growth of hepatocellular carcinoma (HCC) cells and the maintenance of stem cell-like features in HCC remains unclear.

In the present study, we conducted loss-of-function assays of ALDH1 in HCC cells to evaluate the role of ALDH1 in cell growth and the maintenance of stem cell-like features. Moreover, we performed immunohistochemical analyses of HCC surgical specimens to estimate the expression levels of ALDH1 and to examine the relationship between ALDH1 expression and clinicopathological characteristics.

METHODS

Cell culture

HEPATOCELLULAR CARCINOMA CELL lines (Huh1, Huh7, Huh6 and PLC/PRF/5), breast cancer cell lines (MCF-7 and BT-474) and lung cancer cell lines (H460 and H358) were obtained from the Health Science Research Resources Bank (Osaka, Japan) or American Type Culture Collection (Manassas, VA, USA). For the sphere-formation assay of HCC cells, 1000 cells were plated onto ultra-low attachment six-well plates (Corning, Corning, NY, USA), and the number of spheres (>100 μm in diameter) was counted on day 14 of culture.

Reverse transcription polymerase chain reaction (RT–PCR)

RNA extraction and cDNA synthesis were performed as described previously.[14] Quantitative RT–PCR was performed with an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) by using the Universal Probe Library System (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's directions. Primer sequences 5′-AGCCTT CACAGGATCAACAGA-3′ and 5′-TGCAAGGGCTCTTT CCTC-3′ were used for *ALDH1*, and 5′-AAGAGTCCC TGCTACGTGGA-3′ and 5′-CTGGCCACTGTTCATGAA TTT-3′ were used for *ALDH3*. Primer sequences 5′-CT GACTTCAACAGCGACACC-3′ and 5′-TAGCCAAATTCG TTGTCATACC-3′ were used for *GAPDH*. Relative quantification was conducted by using the comparative cycle threshold (Ct) method.

Immunocytochemistry

After fixation with 2% paraformaldehyde and blocking in 10% goat serum, the cells were stained with anti-ALDH1 (BD Biosciences, San Jose, CA, USA), anti-ALDH3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-EpCAM (Abcam, Cambridge, MA, USA) and anti-CD13 (Abcam). Subsequently, the cells were incubated with Alexa-488-conjugated goat antirabbit immunoglobulin G (IgG; Molecular Probes, Eugene, OR, USA) and Alexa-555-conjugated goat antimouse IgG (Molecular Probes). The cells were coverslipped with a mounting medium containing 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Vector Laboratories, Burlingame, CA, USA).

Lentiviral production and transduction

Lentiviral vectors (CS-H1-shRNA-EF-1α-EGFP) expressing short-hairpin RNA (shRNA) that targets human *ALDH1* (target sequences: sh-*ALDH1*-1, 5′-TGTCAAAC CAGCAGAGCAA-3′; sh-*ALDH1*-2, 5′-GGACAATGCTGT TGAATTT-3′), human *ALDH3* (target sequences: sh-*ALDH3*-1, 5′-GCTCAAGAAGTCACTGAAAGA-3′; sh-*ALDH3*-2, 5′-GCAACGACAAGGTGATTAAGA-3′) and *luciferase* were constructed. Recombinant lentiviruses were produced as described elsewhere.[15] The cells were transduced with viruses in the presence of protamine sulfate.

Western blotting

Cells were subjected to western blot analysis using anti-ALDH1 (BD Biosciences), anti-ALDH3 (Santa Cruz Biotechnology) and anti-tubulin (Oncogene Science, Cambridge, MA, USA) antibodies.

Cell proliferation assay

The proliferation of HCC cells expressing shRNA against *ALDH1* or *ALDH3* was examined by using Trypan blue dye staining on days 2 and 4 of culture.

Flow cytometric analysis and sorting

Single-cell suspensions were stained with allophycocyanin-conjugated anti-epithelial cell adhe-

sion molecule (EpCAM) antibody (Biolegend, San Diego, CA, USA). After the incubation, 1 μg/mL of propidium iodide was added to eliminate dead cells. Flow cytometric analyses and sorting were conducted with FACSCanto and FACSAria (BD Biosciences).

Patients and surgical specimens

Forty-nine cancer tissues were subjected to histological examination. The patients comprised 41 men and eight women with an average age of 66 ± 9 years (range, 33–81 years). In addition, 16 diseased livers from liver transplant recipients were also examined. The causes of liver injury were viral cirrhosis ($n = 4$), alcoholic cirrhosis ($n = 4$), primary biliary cirrhosis ($n = 2$), primary sclerosing cholangitis ($n = 2$) and acute/subacute liver failure ($n = 4$). Informed consent for research use of the specimens was obtained for all cases. Paraffin-embedded sections of the tumors and surrounding non-tumor tissues were examined by hematoxylin–eosin (HE) staining and immunohistochemical staining with anti-ALDH1 antibody (BD Biosciences), anti-EpCAM antibody (Abcam) and anti-CD13 (Abcam) antibody. Based on the percentage of HCC cells with high levels of ALDH1 expression, HCC tissues were classified as: no staining (score 0); 1–9% of cells (score 1); 10–24% of cells (score 2); and more than 25% of cells (score 3).

Statistical analysis

Data are presented as the mean ± standard error of the mean. Statistical differences between two groups were analyzed by using the Mann–Whitney *U*-test or χ^2-test. Recurrence-free survival (RFS) was calculated by the Kaplan–Meier method. The prognostic relevance of clinical variables was evaluated by univariate analysis with the log–rank test and by multivariate Cox's regression. Variables associated with RFS in univariate analysis with *P*-values less than 0.20 were retained for multivariate analysis. *P*-values less than 0.05 were considered statistically significant.

RESULTS

Preferential expression of ALDH1 in HCC cells

WE FIRST EXAMINED the mRNA expression of *ALDH1* in various cancer cell lines including HCC cells (Huh1, Huh7, PLC/PRF/5 and Huh6 cells). Quantitative RT–PCR analyses revealed higher expression levels of *ALDH1* in the HCC cell lines than in breast cancer and lung cancer cell lines (Fig. 1a). In addition, immunocytochemical analyses demonstrated varying degrees of ALDH1 expression in HCC cells (Fig. 1b).

Stable knockdown of *ALDH1* in HCC cells

We next conducted lentivirus-mediated *ALDH1*-knockdown experiments. A lentiviral vector expressing shRNA against *luciferase* (sh-*Luc*) was used as a control. We successfully obtained stable cell lines expressing shRNA against *ALDH1* or *luciferase* by cell sorting with enhanced green fluorescent protein as a marker for viral infection. Western blot analysis of these cells showed that two shRNA against *ALDH1* (sh-*ALDH1*-1 and sh-*ALDH1*-2) markedly repressed ALDH1 expression in both cell lines, although sh-*ALDH1*-2 was more effective than sh-*ALDH1*-1 (Fig. 1c). Unexpectedly, neither of the shRNA inhibited the growth of HCC cells as compared to sh-*Luc* (Fig. 1d). We used sh-*ALDH1*-2 in most of the following experiments, but we obtained similar results with sh-*ALDH1*-1 (Fig. 2 and data not shown).

Impact of *ALDH1* knockdown on tumor-initiating HCC cells

We performed a non-adherent sphere assay, a standard approach for evaluating the stem cell features of both normal stem cells and TIC (Fig. 2a). The number of large spheres generated from *ALDH1*-knockdown cells was almost equal to the number of spheres generated from control Huh1 cells and Huh7 cells (Fig. 2b). It has been documented that $EpCAM^+$ cells function as TIC in HCC cells.[16] We examined the expression of EpCAM in view of ALDH1 expression by using flow cytometry. Concordant with the results of the sphere-forming assay, there were no significant differences in the proportion of $EpCAM^{high}$ cells after *ALDH1* knockdown as compared to control Huh1 cells (42.0 ± 3.0% vs 43.4 ± 3.3%) and Huh7 cells (40.5 ± 4.3% vs 40.2 ± 4.0%) (Fig. 2b).

Next, we purified $EpCAM^+$ TIC from Huh1 and Huh7 cell lines by flow cytometry and examined the cell growth and sphere formation ability. Concordant with the results in non-purified HCC cells, *ALDH1* knockdown yielded no significant changes in the proliferative activity or the sphere-forming ability (Fig. 3a,b). To examine whether ALDH1-overexpressing cells co-express other stem cell markers including EpCAM and CD13,[17,18] we performed dual immunostaining of ALDH1 with EpCAM or CD13. Of importance, the immunocytochemical analyses showed no association between the expression of ALDH1 and the expression of

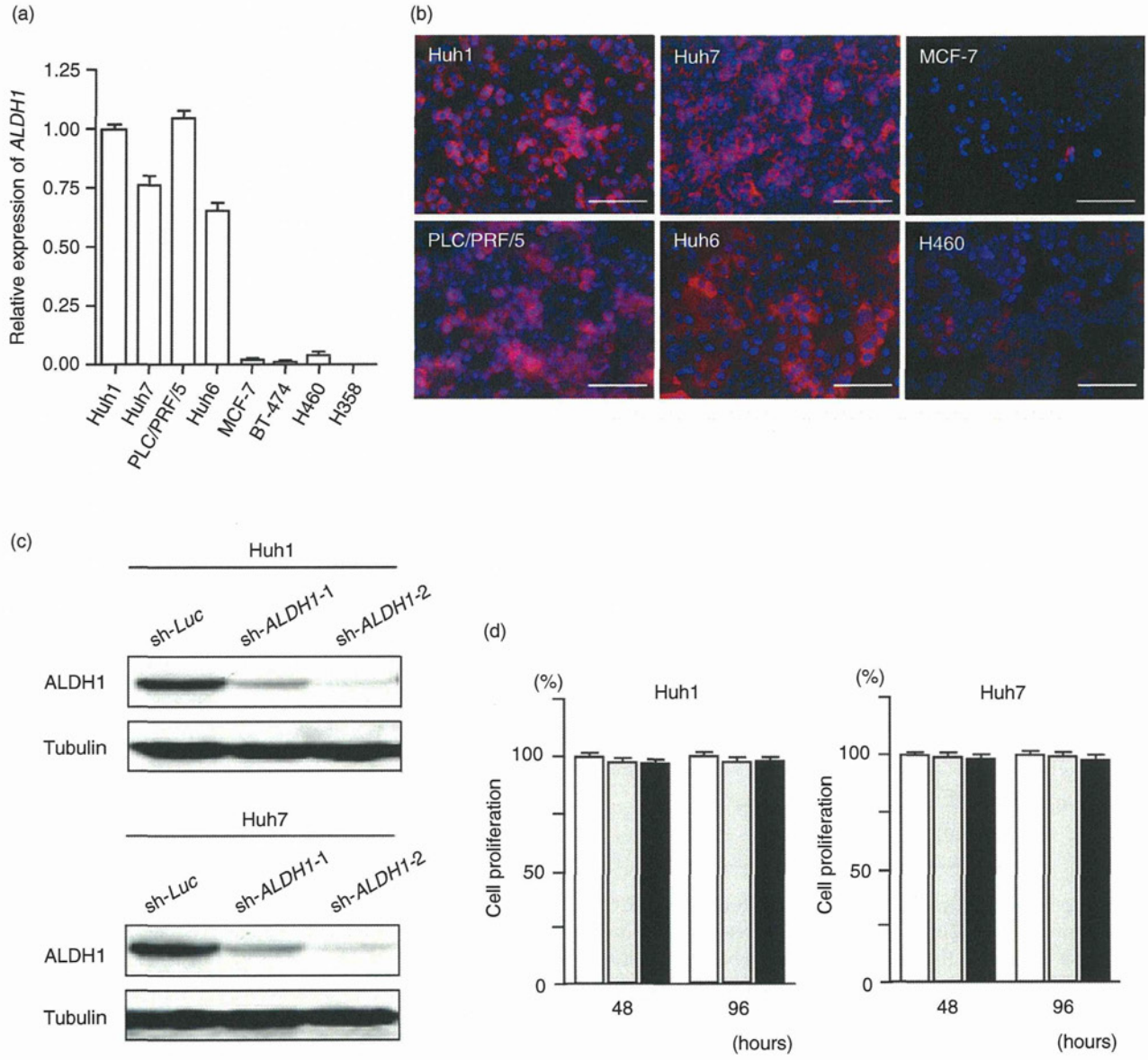

Figure 1 Basal expression and knockdown of *ALDH1* in hepatocellular carcinoma (HCC) cells. (a) mRNA expression of *ALDH1* in various cancer cell lines. (b) Immunocytochemical analyses of aldehyde dehydrogenase 1 A1 (ALDH1) expression in various cancer cells. Nuclear 4′,6′-diamidino-2-phenylindole dihydrochloride staining (blue) and immunofluorescent labeling of ALDH1 (red) are merged. Scale bar = 200 μm. (c) Cells transduced with the indicated lentiviruses were subjected to western blot analysis by using anti-ALDH1 antibody and anti-tubulin antibody (loading control). (d) Cell proliferation in *ALDH1* knockdown HCC cells. The percentages of Huh1 cells stably expressing sh-*Luc*, sh-*ALDH1*-1 and sh-*ALDH1*-2 were 99.6 ± 2.0, 97.5 ± 2.1 and 97.5 ± 1.5 at 48 h, and 99.8 ± 2.2, 97.5 ± 2.2 and 98.5 ± 1.7 at 96 h, respectively. The percentages of Huh7 cells stably expressing sh-*Luc*, sh-*ALDH1*-1 and sh-*ALDH1*-2 were 100.2 ± 1.2, 98.2 ± 2.0 and 98.0 ± 2.0 at 48 h, and 100.8 ± 1.8, 99.4 ± 2.0 and 97.9 ± 2.3 at 96 h, respectively. These results are representative of three independent experiments. □, sh-*Luc*; □, sh-*ALDH1*-1; ■, sh-*ALDH1*-2.

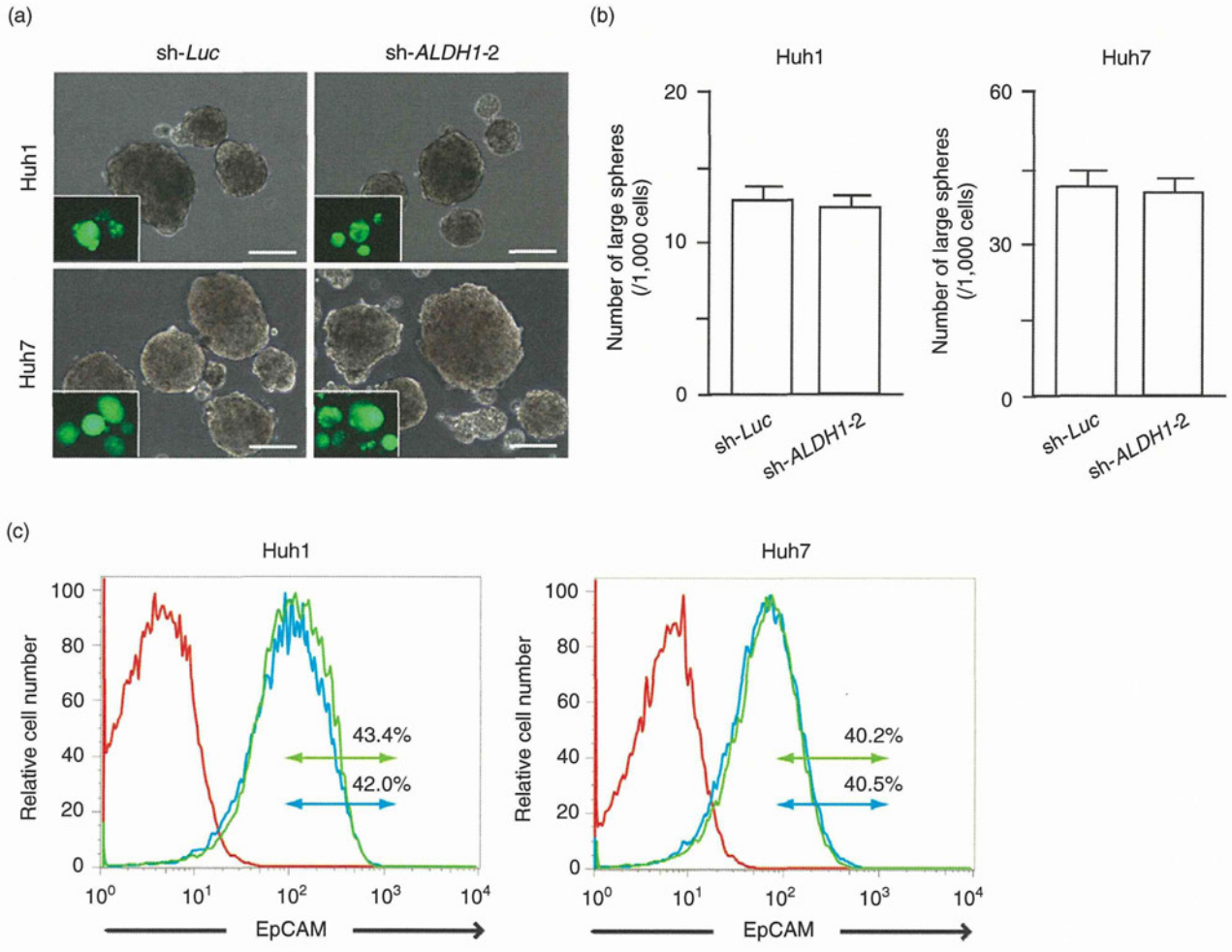

Figure 2 *In vitro* analyses of hepatocellular carcinoma (HCC) cells with sh-*ALDH1*-2-induced *ALDH1* knockdown. (a) Bright-field images of Huh1 and Huh7 cells in non-adherent sphere formation at day 14 of culture. Fluorescence images are shown in the insets. Scale bar = 100 μm. (b) Number of large spheres generated from 1000 Huh1 cells stably expressing sh-*Luc*, sh-*ALDH1*-2 were 12.5 ± 1.2 and 12.1 ± 1.0, respectively. Number of large spheres generated from 1000 Huh7 cells stably expressing sh-*Luc* and sh-*ALDH1*-2 were 40.6 ± 4.5 and 39.8 ± 4.3, respectively. These results are representative of three independent experiments. (c) Flow cytometric profiles in HCC cells after the stable knockdown of *ALDH1*. The percentages of epithelial cell adhesion molecule $(EpCAM)^{high}$ fraction are shown as the mean values for three independent analyses. ▬, Negative control; ▬, sh-*Luc*; ▬, sh-*ALDH1*-2.

EpCAM or CD13 (Fig. 3c). Together, these results indicate that ALDH1 overexpression is not closely associated with stem cell features.

Basal expression and loss-of-function assays of ALDH3 in HCC cells

It has been reported that ALDH3 is highly expressed in lung cancer and breast cancer and that cells expressing a high level of ALDH3 function as CSC in breast cancer.[19,20] We examined the basal levels of ALDH3 expression and conducted loss-of-function assays of ALDH3 in HCC cells. Quantitative RT–PCR analyses and immunocytochemical analyses demonstrated lower levels of ALDH3 expression in HCC cells than in breast cancer and lung cancer cells (Supplementary Figure S1a–c). Because Huh1 cells showed the highest basal expression of ALDH3 among the four HCC cell lines examined, we performed lentivirus-mediated *ALDH3* knockdown in Huh1 cells. Quantitative RT–PCR analysis of cells expressing shRNA against *ALDH3* showed that