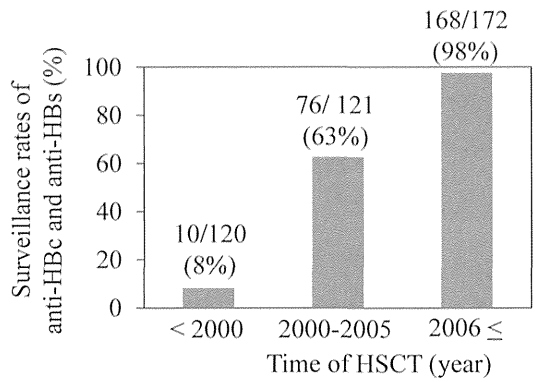
Figure 1. Surveillance rates of antibodies to hepatitis B core antigen (anti-HBc) and to hepatitis B surface antigen (anti-HBs) according to the time of hematopoietic stem cell transplantation (HSCT).



4.4. Definition of HBV Infection Status and HBV Reactivation

In the present study, before HSCT, the recipients were divided into two groups according to HBsAg: HBsAg-positive and HBsAg-negative. Among the HBsAg-negative recipients, anti-HBc-positive and/or anti-HBs-positive recipients were considered as having experienced previous HBV infection [2], and recipients without anti-HBc and anti-HBs were considered as having no previous or current HBV infection.

We defined ≥1 log IU/mL increase in serum HBV DNA from nadir as HBV reactivation in HBsAg-positive recipients. We also defined ≥1 log IU/mL increase, or the re-appearance of HBV DNA from baseline and/or HBsAg, as HBV reactivation in HBsAg-negative recipients.

4.5. Statistical Analysis

Statistical analyses were performed using Statview-J 5.0 (SAS institute, Cary, NC, USA). HBV reactivation rates were calculated by Kaplan-Meier method and evaluated by log-rank test. Baseline was taken as the date of HSCT. $p < 0.05$ was considered statistically significant.

5. Conclusions

HBV reactivation was a common event in HBsAg-positive recipients with HSCT for hematologic malignancies, NUCs are safely and effectively used in these recipients, and extended NUCs therapy may be needed for the prevention of HBV reactivation. In addition, HBV reactivation was occasionally observed in HBsAg-negative recipients with anti-HBc and/or anti-HBs and treated with HSCT, and the immediate use of NUCs could prevent the progression to severe liver damage. Special attention should be paid to recipients with previous exposure to HBV.

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Author Contributions

Shingo Nakamoto, Tatsuo Kanda, Chiaki Nakaseko, Emiko Sakaida, Chikako Ohwada, Masahiro Takeuchi and Yusuke Takeda designed this study. Tatsuo Kanda, Chiaki Nakaseko, Emiko Sakaida, Chikako Ohwada, Masahiro Takeuchi, Yusuke Takeda, Naoya Mimura, Tohru Iseki, Makoto Arai, Fumio Imazeki and Osamu Yokosuka saw patients. Shingo Nakamoto, Tatsuo Kanda, Chiaki Nakaseko, Emiko Sakaida, Chikako Ohwada, Masahiro Takeuchi, Yusuke Takeda, Naoya Mimura, Tohru Iseki, Shuang Wu, Kengo Saito and Hiroshi Shirasawa acquired and analyzed the data. Shingo Nakamoto, Tatsuo Kanda and Chiaki Nakaseko drafted the manuscript; all authors approved the manuscript.

Conflicts of Interest

Tatsuo Kanda reports receiving lecture fees from Bristol-Myers Squibb and GlaxoSmithKline. Osamu Yokosuka reports receiving grant support from Chugai Pharmaceutical, Bayer, MSD, Daiichi-Sankyo, Tanabe-Mitsubishi and Bristol-Myers Squibb. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The other authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (9): Hepatitis B virus

Regulation of microRNA by hepatitis B virus infection and their possible association with control of innate immunity

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Abstract

Hepatitis B virus (HBV) chronically infects more than 350 million people worldwide. HBV causes acute and chronic hepatitis, and is one of the major causes of cirrhosis and hepatocellular carcinoma. There exist complex interactions between HBV and the immune system including adaptive and innate immunity. Toll-like receptors (TLRs) and TLR-signaling pathways are important parts of the innate immune response in HBV infections. It is well known that TLR-ligands could suppress HBV replication and that TLRs play important roles in anti-viral defense. Previous immunological studies demonstrated that HBV e antigen (HBeAg) is more efficient at eliciting T-cell tolerance, including production of specific cytokines IL-2 and interferon gamma, than HBV core antigen. HBeAg downregulates cytokine production in hepatocytes by the inhibition of MAPK or NF-κB activation through the interaction with receptor-interacting serine/threonine protein kinase. MicroRNAs (miRNAs) are also able to regulate various biological processes such as the innate immune response. When the expressions of approximately 1000 miRNAs were compared between human hepatoma cells HepG2 and HepG2.2.15, which could produce HBV virion that infects chimpanzees, using real-time RT-PCR, we observed several different expression levels in miRNAs related to TLRs. Although we and others have shown that HBV modulates the host immune response, several of the miRNAs seem to be involved in the TLR signaling pathways. The possibility that alteration of these miRNAs during HBV infection might play a critical role in innate immunity against HBV infection should be considered. This article is intended to comprehensively review the association between HBV and innate immunity, and to discuss the role of miRNAs in the innate immune response to HBV infection.

Key words: Hepatitis B virus; HepG2.2.15; Innate immunity; MicroRNA; Persistent infection; Toll-like receptor

Core tip: Hepatitis B virus (HBV) is the leading cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma in the world. HBV could interact with the host's innate and adaptive immune responses to establish chronic infection. HBV also interacts with Toll-like receptors (TLRs) and TLR signaling pathways, and regulates host immune responses through the regulation of microRNAs (miRNAs) to some extent. This article fo-

cuses on the involvement of miRNA in the association between HBV and TLR signaling pathways and reviews the miRNAs involved in HBV infection.

Jiang X, Kanda T, Wu S, Nakamura M, Miyamura T, Nakamoto S, Banerjee A, Yokosuka O. Regulation of microRNA by hepatitis B virus infection and their possible association with control of innate immunity. *World J Gastroenterol* 2014; 20(23): 7197-7206 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i23/7197.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i23.7197

INTRODUCTION

Hepatitis B virus (HBV), a member of hepadona viridae, has partially circular double-stranded DNA genome, 3.2 kb in length[1]. It contains four overlapping open reading frames that encode seven proteins: the precore protein, also known serologically as HBe antigen (HBeAg), the core protein (HBcAg), viral polymerase, three forms of the envelope protein known as S antigen (HBsAg) and X (HBx) protein[1,2]. HBV as well as hepatitis C virus (HCV) causes acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC)[3]. Hepatic cirrhosis and HCC are the most common causes of death in patients with chronic liver disease[4].

The outcome of HBV infection is the result of complex interactions between HBV and the immune system including adaptive and innate immunity[5,6]. Toll-like receptors (TLRs) are important parts of the innate immune response in hepatitis virus infections[7]. There are several reports about the important role of TLRs and TLR-mediated signaling in the pathogenesis and outcome of HBV infection[2,5-11].

MicroRNA (miRNA) is one of the endogenous noncoding small RNAs, approximately 18-22 nucleotides in size, a post-transcriptional regulator that binds to the 3'-untranslated region (UTR) of the target gene messenger RNA, usually resulting in cleavage or inhibiting translation of the target gene mRNA[12,13]. It is estimated that the human genome may encode over 2000 miRNAs, which may control about 60% of the human genome[14,15]. Physiologically, miRNAs are able to regulate various biological processes such as cell proliferation, differentiation and apoptosis, neuroprocesses, carcinogenesis and immune response[16-18]. This article is intended to comprehensively review the association between HBV and innate immunity, and to discuss the role of miRNAs in the innate immune response to HBV infection.

INNATE IMMUNITY IS IMPORTANT FOR THE ERADICATION OF HBV

Interferons (IFNs) play an important role in the innate immune response to virus infection. IFN-α and IFN-β (type I IFNs) are secreted by almost all virus-infected cells including hepatocytes and by specialized blood lymphocytes. In contrast, the production of IFN-γ (type II IFN) is restricted to cells of the immune system, such as natural killer (NK) cells, macrophages, and T cells. On the other hand, tumor necrosis factor alpha (TNF-α) primarily initiates innate immune response and triggers acquired immune responses[19]. TNF-α-induced apoptosis is important for clearance of hepatocytes infected with HBV and HCV, and IFN-γ accelerates the killing of theses hepatocytes[19,20]. The previous studies demonstrated that TNF-α and IFN-γ downregulate HBV gene expression in the liver of HBV transgenic mice by post-transcriptionally destabilizing the viral mRNA[21-23]. It has been widely believed that the cytotoxic T lymphocyte response clears viral infections by killing infected cells. However, Chisari's group[21-24] reported that noncytopathic clearance of HBV from hepatocytes by cytokines, which abolish viral replication and HBV gene expression, is another important mechanism. Isogawa *et al*[24] reported that TLR3, TLR4, TLR5, TLR7 and TLR9 ligands could induce antiviral cytokines and inhibit HBV replication in HBV transgenic mice, thereby indicating TLR activation as a powerful strategy for the treatment of chronic HBV infection. HBV replication can be controlled by innate immune response, involving TLRs, if it is activated in hepatocytes[24]. Together, these facts indicate that innate immunity including TLR signaling plays an important role in the pathogenesis of HBV infection.

TOLL-LIKE RECEPTORS AND ANTI-VIRAL DEFENSES

TLRs, germline-encoded pattern recognition receptors (PRRs), can play a central role in host cell recognition and response to various pathogens such as viruses[25]. TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on the cell surface while TLR3, TLR7, TLR8 and TLR9 are expressed within intracellular vesicles. TLR3, TLR7/8 and TLR9 are involved in the recognition of viral nucleotides such as double-stranded RNA, single-stranded RNA and DNA, respectively[26]. Other than TLRs, membrane-bound C-type lectin receptors (CLRs), cytosolic proteins such as NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs), which include retinoic acid-inducible gene I (RIG- I), melanoma differentiation antigen 5 (MDA5), and lipophosphoglycan biosynthetic protein 2 (LPG2), and unidentified proteins that mediate sensing of cytosolic DNA or retrovirus infection, are also involved in the recognition of pathogen-associated molecular patterns (PAMPs)[25].

TLRs play a crucial role in defending against pathogenic infection through the induction of inflammatory cytokines and type I IFNs by myeloid differentiation primary response 88 (MYD88)-dependent and MYD88-independent pathway. In the MYD88-dependent pathway, MYD88 recruits a set of signal cascades such as MAPK and NF-κB through receptor-interacting serine/

threonine protein kinase (RIPK/RIP). In the MYD88-independent pathway, TLR3 activates NF-κB and MAPKs through RIPK. TLR3 also activates IFN regulatory factor 3 (IRF3) and IRF7 *via* TRIF/TICAM-1, inducing the production of type I IFN. The activated NF-κB and IRFs are translocated to the nucleus. NF-κB and MAPKs initiate the transcription of inflammatory cytokine genes, whereas IRFs initiate the transcription of type I IFN[2]. RIG- I and MDA5 pathways can also activate IRF3 to produce type I IFNs. RNA helicases RIG- I and MDA5, specific receptors for double-stranded RNA, and the downstream mitochondrial effector known as CARDIF/MAVS/VISA/IPS-1, are also major pathways for type I IFN induction.

ASSOCIATION BETWEEN HBV AND TOLL-LIKE RECEPTORS

TLRs have been recognized as playing an important role in the pathogenesis of chronic hepatitis B[8]. NF-κB is activated by three TLR adaptors, MYD88, Toll/interleukin (IL)-1 receptor (TIR)-domain-containing adaptor-inducing IFNβ (TRIF), and IFN promoter stimulator 1 (IPS-1), to elicit anti-HBV response in both HepG2 and Huh7 cells[27]. Down-regulations of TLR7 and TLR9 mRNA were observed in peripheral blood mononuclear cells (PBMC) of HBV-infected patients[28]. Chen *et al*[29] reported that TLR1, TLR2, TLR4 and TLR6 transcripts were also downregulated in PBMC of chronic hepatitis B patients. After being challenged by TLR2 and TLR4 ligands, cytokine production was impaired in PBMC of chronic hepatitis B patients on the basis of the levels of plasma HBsAg[29]. Xie *et al*[30] reported that HBV infection results in reduced frequency of circulating plasmacytoid dendritic cells (pDCs) and their functional impairment *via* inhibiting TLR9 expression. HBV replication suppresses the TLR-stimulated expression of pro-inflammatory cytokines (TNF, IL6) and the activation of IRF3[31]. It has also been reported that HBV could target RIG- I signaling by HBx-mediated IPS-1 down-regulation, thereby attenuating the antiviral response of the innate immune system[32].

HBV E ANTIGEN DOWNREGULATES CYTOKINE PRODUCTION

The HBV precore/core region of HBV genome also encodes HBeAg as well as the HBV core. The precore stop codon prevents the formation of precore protein and HBeAg[2,33]. The existence of HBeAg in serum is known to be a marker of a high degree of viral infectivity. In Japan, the major HBV genotypes are B and C, but our previous study[34] revealed that the precore mutation A1896 and the core promoter mutations at nt1762 and 1764 were found more frequently in acute liver failure than in acute hepatitis, and HBV genotype B was predominant in acute liver failure. It has also been shown that acute liver failure occasionally occurs in persons who are negative for HBeAg[35,36]. It is well known that perinatal transmission of HBV occurs in about 10%-20% of HBeAg-negative mothers without prevention of perinatal HBV transmission by combined passive and active immunoprophylaxis, and the babies are at risk of developing fulminant hepatitis[37]. Chronic hepatitis B with high HBV DNA and ant-HBe is associated with a severe and evolutive liver disease[38]. These clinical findings could be assumed to have immune tolerance for HBeAg, although the function of HBV precore or HBeAg is unknown. Previous immunological studies[39-41] demonstrated that HBeAg is more efficient at eliciting T-cell tolerance, including production of its specific cytokines IL-2 and IFN-γ, than HBV core antigen. We also demonstrated that HBeAg expression inhibits IFN and cytokine production[2] and that HBeAg physically associates with RIPK2 and regulates IL-6 gene expression[6]. Visvanathan *et al*[42] reported that the expression of TLR2 on hepatocytes, Kupffer cells, and peripheral monocytes was significantly reduced in HBeAg-positive chronic hepatitis B patients. Thus, HBV seems to have evolved strategies that block the effector mechanisms induced through IFN and/or cytokine signaling pathways, similar to other viruses[19].

MIRNAS WERE DIFFERENTIALLY EXPRESSED IN HEPG2.2.15 AND HEPG2

HepG2.2.15 cells assemble and secrete HBV virion that infects chimpanzees[43,44]. We examined the expression of approximately 1000 miRNAs in the human hepatoma cells HepG2.2.15 and HepG2 using real-time RT-PCR, the most sensitive technique for mRNA detection and quantification[45,46].

First, 1008 miRNAs were examined in the hepatoma cells HepG2.2.15 and HepG2, using quantitative real-time RT-PCR with specific primers (Qiagen, Hilden, Germany). SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A and RNU6-2 were used as endogenous controls to normalize expression to determine the fold-change in miRNA expression between the test sample (HepG2.2.15) and control sample (HepG2) by 2-ddCT (comparative cycle threshold) method[21]. MiRNAs were annotated by Entrez Gene (NCBI, Bethesda, MD, United States), accessed on 2/27/2013. Data were analyzed with miRNA PCR array data analysis software (http://www.sabiosciences.com/mirnaArrayDataAnalysis.php). Scatter plot analysis is shown in Figure 1A. There were differences in expression between HepG2 and HepG2.2.15 (Figure 1B).

We then excluded 599 miRNAs according to the following criteria: (1) average threshold cycle was relatively high (> 30) in either HepG2 or HepG2.2.15, and was reasonably low in the other samples (< 30); (2) average threshold cycle was relatively high (> 30), meaning that its relative expression level was low, in both HepG2 and

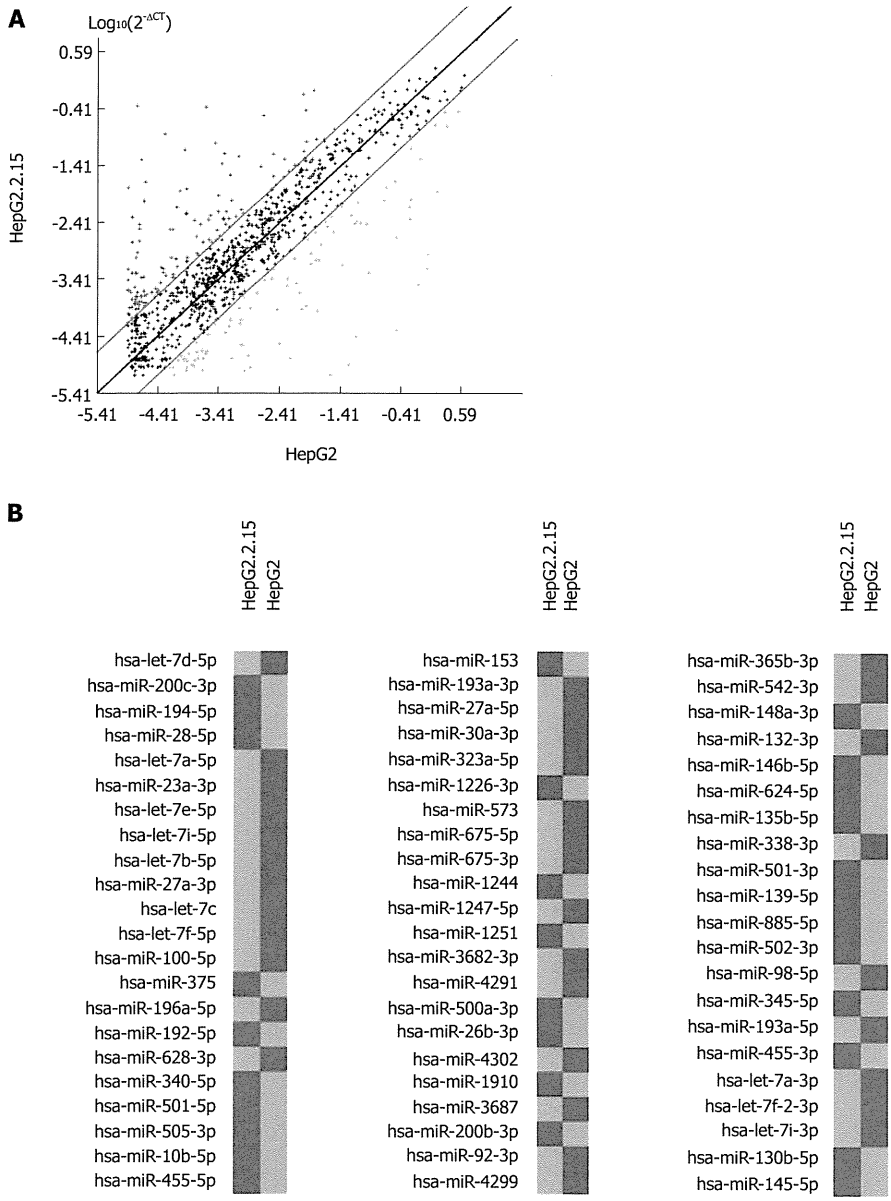

Figure 1 MicroRNAs expression in hepatoma cells HepG2.2.15 and HepG2. A: Scatter plots of 1008 miRNAs indicate 2^{-dCT} numerical values in HepG2 cells (x-axis) and HepG2.2.15 cells (y-axis). The black line indicates fold changes of 1. The pink lines indicate 5-fold change in miRNA expression threshold, comparing HepG2.2.15 with HepG2. Red + indicates miRNA expressed at least 5-fold higher in HepG2.2.15 than in HepG2 cells. Green + indicates miRNA expressed at least 5-fold lower in HepG2.2.15 than in HepG2 cells. Black + indicates that the difference of miRNA between the two cells was within 5-fold; B: Comparison of miRNAs expression between HepG2 and HepG2.2.15 cells. Red color indicates miRNA expressed at least 5-fold higher in HepG2.2.15 than in HepG2 cells. Green color indicates miRNA expressed at least 5-fold lower in HepG2.2.15 than in HepG2 cells. miRNAs: MicroRNAs.

HepG2.2.15; and (3) average threshold cycle was either not determined or was greater than the defined cut-off value (default 35) in both samples, meaning that its expression was undetected, making this fold-change result erroneous and uninterpretable.

Out of 409 miRNAs examined, 30 (7.3%) were upregulated by 5-fold or greater in HepG2.2.15 compared to HepG2. Twelve miRNAs (miR-200b-3p, miR-505-3p, miR-148a-3p, miR-145-5p, miR-194-5p, miR-885-5p, miR-192-5p, miR-146b-5p, miR-340-5p, miR-375, miR-139-5p and miR-200c-3p) were upregulated 10-fold or more in HepG2.2.15 cells. MiRNAs upregulated 5-fold or more are shown in Figures 1B and 2A. On the other hand, out of 409 miRNAs, 35 (8.6%) were downregulated 5-fold or more in HepG2.2.15 compared to HepG2. Twenty-two miRNAs (let-7c, miR-573, let-7b-5p, miR-338-3p, miR-100-5p, miR-92b-3p, miR-542-3p, miR-4302, miR-4291, miR-193a-5p, miR-98-5p, miR-4299, miR-132-3p, let-7f-2-3p, let-7f-5p, let-7i-5p, let-7d-5p, miR-193a-3p, let-7a-5p, let-7i-3p, miR-196a-p and let-7a-3p) were downregulated 10-fold or more in HepG2.2.15 cells. MiRNAs downregulated 5-fold or

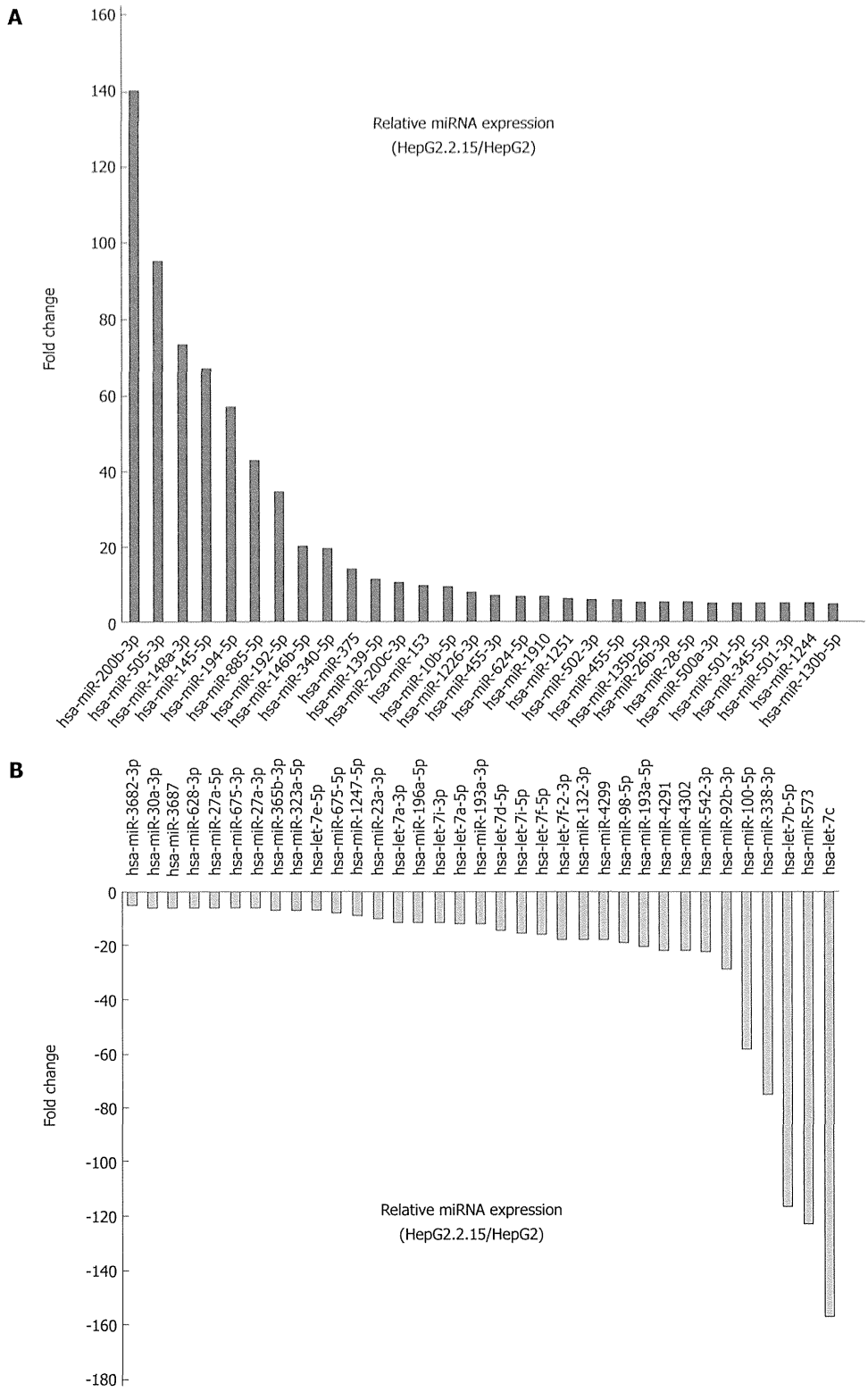

Figure 2 MicroRNAs expressed at more than 5-fold difference between hepatoma cells. HepG2.2.15 and HepG2 cells. A: MiRNA expressed at least 5-fold higher in HepG2.2.15 than in HepG2 cells; B: MiRNA expressed at least 5-fold lower in HepG2.2.15 than in HepG2 cells. miRNAs: MicroRNAs.

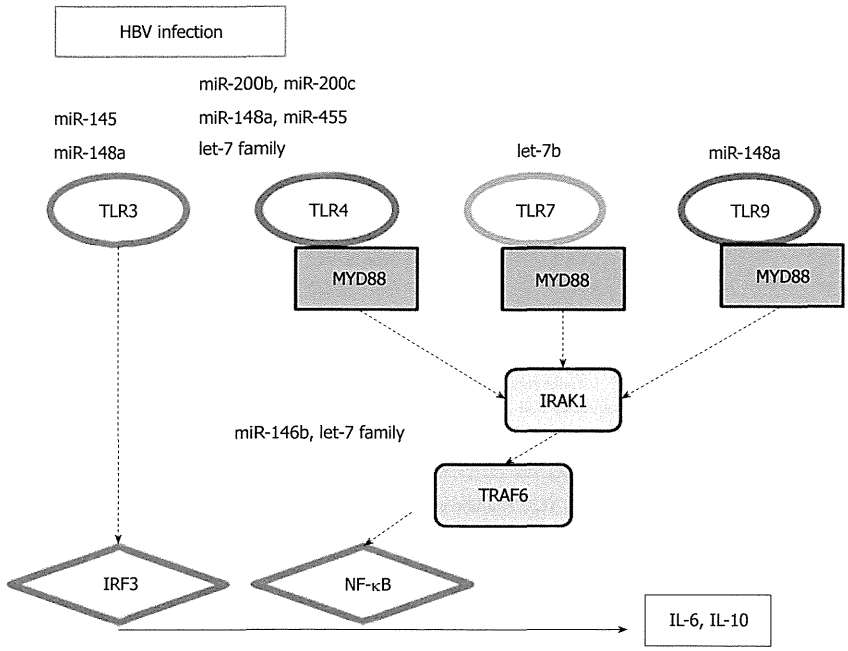

Figure 3 MicroRNAs and Toll-like receptor signaling pathway in hepatitis B virus infection. IRAK: Interleukin (IL)-1 receptor-associated kinase 1; IRF: Interferon regulator factor; miRNA: MicroRNA; MYD88: Myeloid differentiation factor 88; NF-κB: Nuclear factor-κB; TLR: Toll-like receptor; TRAF: Tumor necrosis factor receptor-associated factor.

Table 1 MicroRNAs associated with Toll-like receptor signaling pathways, upregulated by 5-fold or greater in HepG2.2.15 than in HepG2

MicroRNAs	Genomic location	Fold changes	Description of target molecules/pathways	Ref.
miR-200b-3p	1p36.33	140.15	TLR4 signaling through MyD88-dependent pathway	[47]
miR-148a-3p	7p15.2	73.36	TLR3, TLR4 and TLR9 agonists upregulated miR-148/152 expression	[48]
miR-145-5p	5q32	66.97	miR-145 promoted interferon-β induction by SOCS7	[49]
miR-146b-5p	10q24.32	20.05	TNF receptor-associated factor 6 and IL-1 receptor-associated kinase 1	[50]
miR-200c-3p	12p13.31	10.75	TLR4 signaling through MyD88-dependent pathway	[47]
miR-455-3p	9q32	7.36	miR-455 was involved in TLR4 signaling pathway through E2F1 transcription factor	[51]
miR-455-5p	9q32	5.76	miR-455 was involved in TLR4 signaling pathway through E2F1 transcription factor	[51]

Genomic location was analyzed using GeneCards (http://gene4.weizmann.ac.il/). TLR: Toll-like receptor.

more are shown in Figures 1B and 2B.

MIRNAS RELATED TO TLR PATHWAY UPREGULATED IN HEPG2.2.15 CELL LINES

Innate immunity represents the first line of defense against HBV, and we and others have reported its importance in the persistence of HBV infection[2,5-11]. So, we focused on miRNAs related to the TLR pathway. Among miRNAs upregulated 5-fold or more in HepG2.2.15 cells, 7 miRNAs (miR-200b-3p, miR-148a-3p, miR-145-5p, miR-146b-5p, miR-200c-3p, miR-455-3p and miR-455-5p) were reported to be related to TLR pathways (Table 1). MiRNAs miR-200b and miR-200c are the factors that modify the efficiency of TLR4 signaling through MYD88 in HEK293 cells[47]. TLR3, TLR4 and TLR9 agonists upregulated miR-148/152 expression and downregulated calcium/calmodulin-dependent protein kinase II (CaMK II) in dendritic cells (DCs) on maturation[48]. Thus miR-148/152 can act as fine-tuners in regulating the innate response and antigen-presenting capacity of DCs[48]. Exogenous miR-145 promoted IFN-β induction by targeting the suppressor of cytokine signaling 7 (SOCS7), through the nuclear translocation of signal transducer and activator of transcription 3 (STAT3) and SOCS7-silencing enhanced IFN-γ induction by stimulation with TLR3 ligand, poly(I-C)[49]. MiR-146 plays a role in the control of TLR and cytokine signaling through a negative feedback regulation loop involving down-regulation of interleukin (IL)-1 receptor-associated kinase 1 (IRAK1) and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) protein levels[50]. MiR-455 was involved in the TLR4 signaling pathway through E2F1 transcription factor[51].

Table 2 MicroRNAs associated with Toll-like receptor signaling pathways, downregulated by 5-fold or greater in HepG2.2.15 than in HepG2

MicroRNAs	Genomic location	Fold changes	Description of target molecules/pathways	Ref.
let-7e-5p	19q13.33	-7.29	Akt1 activated by TLR4-ligand LPS, positively regulated let-7e	[52]
let-7a-3p	9q22.32 11q24.1 22q13.31	-11.44	Repression of let-7 family relieves IL-6 and IL-10 mRNAs from negative post-transcriptional control in TLR4 signaling pathway	[53]
let-7i-3p	12q14.1	-11.57	let-7i regulates Toll-like receptor 4 expression	[54]
let-7a-5p	9q22.32 11q24.1 22q13.31	-11.96	Repression of let-7 family relieves IL-6 and IL-10 mRNAs from negative post-transcriptional control in TLR4 signaling pathway	[53]
let-7d-5p	9q22.32	-14.03	Repression of let-7 family relieves IL-6 and IL-10 mRNAs from negative post-transcriptional control in TLR4 signaling pathway	[53]
let-7i-5p	12q14.1	-15.10	let-7i regulates Toll-like receptor 4 expression	[54,55]
miR-132-3p	17p13.3	-18.18	TNF receptor-associated factor 6 and IL-1 receptor-associated kinase 1	[56]
let-7b-5p	22q13.31	-116.31	let-7b activates TLR 7	[56]

Genomic location was analyzed using GeneCards (http://gene4.weizmann.ac.il/). TLR: Toll-like receptor.

MIRNAS RELATED TO TLR PATHWAY DOWNREGULATED IN HEPG2.2.15 CELL LINES

Among miRNAs downregulated 5-fold or more in HepG2.2.15 cells, 8 miRNAs (let-7e-5p, let-7a-3p, let-7i-3p, let-7a-5p, let-7d-5p, let-7i-5p, miR-132-3p and let-7b-5p) were reported to be related to TLR pathways (Table 2). Protein kinase Akt1, which is activated by the TLR4-ligand lipopolysaccharide (LPS), positively regulated let-7e and miR-181c but negatively regulated miR-155 and miR-125b[52]. Repression of the let-7 family relieves IL-6 and IL-10 mRNAs from negative post-transcriptional control in the TLR4 signaling pathway[53], and the miRNAs let-7i and let-7b activate TLR4 and TLR7, respectively[54-56].

ROLE OF MIRNAS IN REGULATION OF INNATE IMMUNE RESPONSE IN HBV INFECTION

In the present study, 30 and 35 miRNAs were upregulated and downregulated, respectively, by 5-fold or greater in HepG2.2.15 compared to its parental cell line HepG2. These results indicate that miRNAs could play an important role in chronic persistent HBV infection. Su *et al*[57] reported that miR-155 enhances innate antiviral immunity through promoting the JAK/STAT signaling pathway by targeting SOCS1, inhibiting HBV replication. The possibility cannot be ruled out that HBV persistently infects hepatocytes through the regulation of miRNAs.

We also speculated that several of the miRNAs involved in the TLR signaling pathway play a critical role in innate immunity against HBV infection[5,24] (Figure 3). It has been reported that miR-21[58], miR-22[59,60], miR-122[58], miR-194[61] and miR-219-1[62] are associated with chronic persistent HBV infection as well as its clearance. In the present study, miR-194 was upregulated 10-fold or more in HepG2.2.15 cells.

CONCLUSION

MicroRNAs miR-122 and miR-130a play an important role in chronic hepatitis C[63,64]. Regulation of miRNAs also plays an important role in HIV infection[65]. In HCV infection, a set of miRNAs that regulate host immune response are modulated[66]. We and others have demonstrated that HBV modulates the host immune response. It might be possible that HBV as well as HCV regulates host immune response through the regulation of miRNAs in some steps toward chronic infection. MiRNAs and their regulation play a critical role in HBV infection, and HBV may regulate the TLR signaling pathway through the regulation of miRNAs.

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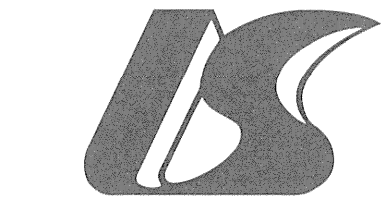

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Lipopolysaccharide blocks induction of unfolded protein response in human hepatoma cell lines

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ABSTRACT

In the present study, we examined whether unfolded protein response (UPR) determined the hepatic cell damage induced by an innate immune response including TLR signaling pathways. We observed that lipopolysaccharide (LPS) transcriptionally downregulates 78-kDa glucose-regulated protein/immunoglobulin heavy-chain binding protein (GRP78/Bip), known to confer resistance to apoptosis. We also observed that LPS blocked the induction of UPR and led to poly(ADP-ribose) polymerase (PARP) cleavage in hepatocytes. We also demonstrated that overexpression of GRP78 rescued HepG2 cells treated with LPS from PARP cleavage. These data suggest that UPR downregulation could be a collateral effect of the LPS treatment. We speculate that UPR is an important factor of hepatic cell damage induced by an innate immune response.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the major health problems worldwide [1]. Most patients with early-stage HCC are treated with potentially curative therapies (resection, liver transplantation, or local ablation either by radiofrequency or percutaneous ethanol injection) that have 5-year survival rates of 50–70% [1–3]. The survival of patients with Child's A or Child's B status is better than with Child's C disease. Despite successful resection, the remnant cirrhotic liver frequently develops new HCC lesions, seriously curtailing long-term survival [4]. Although it has recently been reported that sorafenib is effective for the treatment of advanced HCC [5,6], we might still require better understanding of the mechanism of the development of HCC as well as more therapeutic options for HCC patients.

The endoplasmic reticulum (ER) is an elaborate cellular organelle essential for cell function and survival [7]. The ER unfolded protein response (UPR) restores equilibrium to ER [8]. ER chaperone, 78-kDa glucose-regulated protein (GRP78/BiP), is a central regulator of ER homeostasis and has anti-apoptotic properties [9,10]. GRP78 promotes tumor proliferation, survival, metastasis and resistance to a wide variety of therapies [9]. UPR is a concerted, complex cellular response that is mediated through three ER transmembrane receptors: pancreatic ER kinase or PKR-like ER kinase (PERK), activating transcription factor-6 (ATF6) and inositol-requiring enzyme 1 (IRE1) [7]. IRE1 drives the expression of the pro-survival factor X-box binding protein-1 (XBP1), a hallmark of UPR induction [11]. Although the role of C/EBP homologous protein (CHOP) in apoptosis is controversial, this transcription factor, mediating apoptosis, may exert a tissue-specific protective function [12]. In resting cells, all three receptors are maintained in an inactive state through association with GRP78. Under the conditions of ER stress, accumulating unfolded proteins lead to GRP78 dissociation and activation of the three ER stress receptors triggering UPR. UPR is a pro-survival response aimed at reducing the backlog of unfolded proteins and restoring normal ER function. However, if the stress cannot be resolved, this protective signaling switches to a pro-apoptotic response. Failure to induce UPR also leads to apoptotic cell death [13–15].

Toll-like receptor (TLR) 4 recognizes lipopolysaccharide (LPS), a major cell wall component of gram-negative bacteria that activates the innate immune system. Recognition of LPS requires CD14 in addition to TLR4. The responsiveness of the TLR4 and CD14 complex to LPS is enhanced by MD2 [16]. A condition of prolonged ER stress occurs during the response of the host to invasive organisms, as

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exemplified by exposure of cells to LPS, which activates TLR4 signaling through MyD88-Mal and TRIF-TRAM adaptors. This activation results in the production of inflammatory cytokines and antimicrobial proteins [8,17]. Human hepatocytes also express TLR4 [18].

It has been shown that combining drugs capable of suppressing GRP78 with conventional agents might represent a novel approach for eliminating residual tumor cells [19], and also that ATF6, XBP1 and GRP78 genes are activated in human HCC [20]. We and others have also previously observed LPS-induced apoptosis in hepatoma cell lines [18,21,22]. In the present study, we examined the effects of LPS on UPR in hepatocytes during the process of LPS-induced apoptosis. Our study revealed that UPR determined the hepatic cell damage induced by an innate immune response including TLR signaling pathways.

2. Materials and methods

2.1. Plasmids, cells and reagents

Plasmids pFLAG/CMV2 and pFLAG-human GRP78 vectors were generously provided by Prof. Kim WU (Catholic University of Korea, Seoul, South Korea) [15]. Cells (5×10^5) were transfected with 0.2 μg of plasmid pFLAG/CMV2 or pFLAG-human GRP78 vectors using Effectene (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Human hepatoma cells (HepG2, PLC/PRF/5 and Huh7) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 100 U/mL of penicillin G and 100 μg/mL of streptomycin at 37 °C in a 5%-CO_2 incubator. HepG2 or Huh7 cells were plated in 6-well plates and incubated with agonists of TLR1/TLR2 (Pam3CSK4.3HCL, 0.1 μg/mL), TLR3 (Poly[I–C], 50 μg/mL), TLR4 (LPS derived from *Escherichia coli*, 500–1000 ng/mL), TLR5 (purified Flagellin, 100 μg/mL), TLR6/TLR2 (MALP-2, 100 μg/mL), TLR7 (Imiquimod [R-837], 2.5 μg/mL), and TLR9 (type B CpG ODN, 0.5 μg/mL) (all purchased from Imgenex Corp., San Diego, CA). To remove LPS and eliminate the possibility of LPS contamination, we treated LPS with polymyxin B sulfate (Wako Pure Chemical Industries, Osaka, Japan), an LPS-induced TLR4 activation inhibitor [23].

2.2. RNA purification and real-time RT-PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen). Five micrograms of RNA was reverse-transcribed with the PrimeScript RT reagent (Perfect Real Time; Takara, Otsu, Japan). PCR amplification was performed on cDNA templates using primers specific for glucose-regulated protein 78 kDa (GRP78) (sense primer 5′-GCCTGTATTTCTAGACCTGCC-3′ and antisense primer 5′-TTCATCTTGCCAGCCAGTTG-3′), for X-box binding protein 1 (XBP1) (sense primer 5′-AATGAAGTGAGGCCAGTGG-3′ and antisense primer 5′-TCAATACCGCCAGAATCCATG-3′), for C/EBP homologous protein (CHOP) (sense primer 5′-TTAAGTCTAAGGCACTGAGCGTATC-3′ and antisense primer 5′-TGCTTTCAGGTGTGGTGATG-3′) and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense primer 5′-ACCCACTCCTCCACCTTTG-3′ and antisense primer 5′-CTCTTGTGCTCTTGCTGGG-3′). For RNA quantification, real-time PCR was performed using SYBR Green I (StepOne real-time PCR system; Applied Biosystems, Forester City, CA) following the manufacturer's protocol. Data analysis was based on the ΔΔCt method. The expressions of the genes of interest were normalized to the expression of GAPDH.

2.3. Western blotting

Cells were harvested using sodium dodecyl sulfate sample buffer. Four to 10 μg of proteins was subjected to electrophoresis on 5–20% polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (ATTO Bio Instrument, Tokyo, Japan). Membranes were probed with antibodies specific for GRP78, CHOP, IRE1α, and poly(ADP-ribose) polymerase (PARP) (Cell Signaling Technology, Danvers, MA), and XBP-1 and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA). After washing, membranes were incubated with secondary HRP-conjugated antibodies. Signals were detected by enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK) and scanned by image analyzer LAS-4000 and Image Gauge (Fuji Film, Tokyo, Japan).

2.4. Cell proliferation assay

To evaluate cell viability, CellTiter 96 AQ One Solution Cell Proliferation Assay (MTS assay; Promega, Madison, WI) was used as previously described [18]. In brief, 1×10^3 cells/100 μL were seeded onto 96-well plates. After 24 h, LPS was added and incubated for 48 h in 5% CO_2 at 37 °C. Twenty microliters/well of the MTS reagent was added to 100 μL of media containing cells in each well of 96-well plates, and left for 4 h at 37 °C in a humidified 5%-CO_2 atmosphere. For analysis, absorbance at 490 nm was measured using a Bio-Rad iMark microplate reader (Bio-Rad, Hercules, CA). Color reactions of MTS from medium without cells were used as non-specific background. The percentages of surviving cells from each group relative to untreated control groups, defined as 100% survival, were determined by reduction of MTS.

2.5. Apoptosis

APOPercentage Apoptosis Assay (Biocolor Belfast, Northern Ireland) was used to quantify apoptosis according to the manufacturer's instructions. Transfer and exposure of phosphatidyl serine to the exterior surface of the membrane has been linked to the onset of apoptosis. Phosphatidyl serine transmembrane movement results in the uptake of APOPercentage dye by apoptosis-committed cells. Purple-red stained cells were identified as apoptotic cells using light microscopy. The number of purple-red cells/300 cells was counted as previously described [18].

2.6. Statistical analysis

Data were expressed as mean ± standard deviation (SD). Differences were evaluated by Student's *t*-test. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using DA Stats software (O. Nagata, Nifty Serve: PAF01644).

3. Results

3.1. LPS reduced GRP78 mRNA levels in human hepatoma cell lines

Previous studies reported that LPS induced ER stress in the liver and hepatocytes [24–26], and we examined the effects of LPS on ER stress in liver cancer cells. Inflammatory response was recently reported to induce ER stress and UPR, and the latter recovers proper ER function or activates apoptosis [26]. It is well known that GRP78 is a key protein triggering UPR [7,27] and that GRP78 expression is elevated in human HCC [20]. To examine the effects of TLR ligands on ER stress signaling in hepatocytes, we treated HepG2 with TLR1-9 ligands and analyzed GRP78 mRNA by real-time RT-PCR 24 h later (Fig. 1A and B). Mean ΔCt ± standard deviation was 5.4 ± 0.034 and 6.1 ± 0.051 in HepG2 treated with Pam3CSK4.3HCL

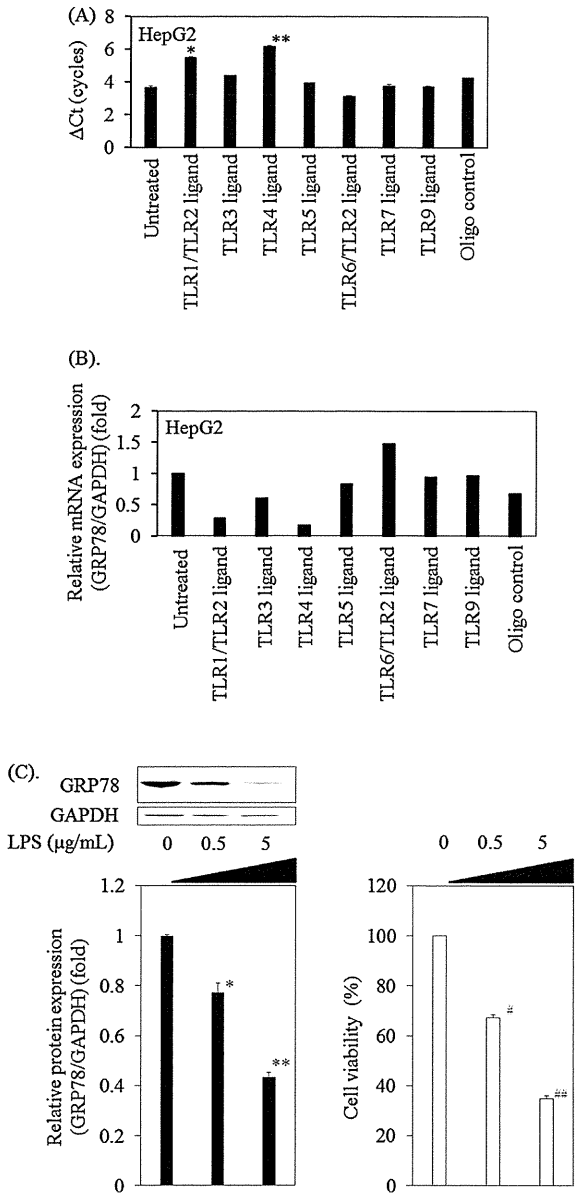

Fig. 1. Effects of TLR ligands on 78-kDa glucose-regulated protein (GRP78) mRNA expression in human hepatoma HepG2 cells. HepG2 cells (A, B) were cultured for 24 h with ligands of Toll-like receptor (TLR)1/TLR2 (Pam3CSK4), TLR3 (Poly[I–C]), TLR4 (LPS), TLR5 (Flagellin), TLR6/TLR2 (MALP-2), TLR7 (Imiquimod [R-837]), TLR9 (type B CpG oligonucleotide) and control oligonucleotide (CpG oligonucleotide as control) as indicated in Section 2. GRP78 mRNA expression was examined by real-time RT-PCR. GAPDH was used for normalization. (A) Statistical analysis of GRP78 mRNA expression in TLR ligand-treated and untreated cellular RNA by real-time RT-PCR by ΔCt. Data are expressed as mean ± SD of triplicate determinations from 1 experiment representative of 3 independent experiments. *P = 0.020 and **P = 0.030, compared to untreated control by Student's t-test. (B) Fold regulation is expressed as real-time RT-PCR based on $2^{-\Delta\Delta Ct}$ method. (C) Effects of TLR4 ligand LPS on GRP78 protein expression and cell viability in HepG2 cells. Cells were cultured for 48 h with or without TLR4 ligand (LPS, 500 ng/mL or 5 μg/mL). Ten micrograms of proteins was subjected to electrophoresis on 5–20% gradient polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. GRP78 protein expression was examined by Western blot. GAPDH was used for normalization. *P = 0.00046 and **P = 0.0000075, compared to untreated control by Student's t-test. Cell viability was examined by MTS assay. #P = 0.000001 and ##P = 0.000000038, compared to untreated control by Student's t-test. Data are expressed as mean ± SD of triplicate determinations from 1 experiment representative of 3 independent experiments.

and LPS, respectively (n = 3, P = 0.020 and n = 3, P = 0.030, Fig. 1A). These results indicated that the stimulation by Pam3CSK4.3HCL and LPS, respectively, inhibited the GRP78 mRNA levels of HepG2 cells (27.9 ± 3.4% and 17.6 ± 5.1% compared with untreated control) (Fig. 1B). Other TLR ligands did not significantly alter the GRP78 mRNA expression of HepG2 (Fig. 1A and B). As HepG2 cells do not express TLR2 [17], we focused on LPS (TLR4 ligand). LPS did not significantly differently reduce GRP78 mRNA levels of Huh7 cells (71.5 ± 3.9% compared with untreated cells, data not shown). Thus, in contrast to HepG2, the change in GRP78 mRNA expression of Huh7 was milder in our experimental conditions (data not shown). We previously reported that LPS induced apoptosis in hepatoma cell lines [18]. In the present study, we examined the effects of 500 ng/mL or 5 μg/mL of LPS on GRP78 protein expression and cell viabilities of HepG2 cells (Fig. 1C). GRP78 expression and cell viability were downregulated according to LPS doses. Five hundred nanograms per milliliter of LPS reduced cell viability in HepG2 (67.4 ± 1.0%; n = 3, P = 0.024) and in PLC/PRF/5 cells (63.9 ± 12.9%; n = 3, P = 0.0013), but had very little effect on Huh7 cells (94.0 ± 3.4%; n = 3, P = 0.16). To inhibit or remove LPS initiating TLR4-dependent signaling, we incubated 0.5 μg of LPS with 100 μg of polymyxin B sulfate for 0.5 h at 25 °C, then added them to HepG2 cells (concentration of LPS 0.5 μg/mL), but we did not observe any changes of cell viabilities by 48 h (108 ± 8.8%; n = 3, P = 0.19). This result suggested that LPS specifically reduced HepG2 cell viability. Based on these results, we mainly used the HepG2 cell line, and 0.5 μg was selected as LPS dose for further analysis in the present study.

3.2. LPS induces cleavage of PARP in human hepatoma cell lines

Cleavage of the DNA repair enzyme PARP from 116 kDa protein to a signature 86 kDa fragment is associated with a variety of apoptotic responses. PARP is a nuclear protein and a downstream substrate of activated caspase-3, that is, PARP cleavage is a hallmark of caspase-3 activation. To investigate whether treatment of human hepatoma HepG2 cells with LPS induces PARP cleavage, a hallmark of apoptosis, HepG2 cells were incubated with LPS or with LPS plus ethanol. Whole cellular proteins were collected after 24 h of LPS treatment, were subjected to SDS-PAGE, and were then analyzed by Western blot analysis using a specific antibody (Fig. 2A). Cells treated with LPS or with LPS plus ethanol displayed significant cleavage of the native 116 kDa PARP to its 86 kDa signature peptide. In contrast, untreated control cells did not display detectable PARP cleavage (Fig. 2A–C), which was similar to the previous observation [18]. There are two important BH3-only proteins associated with ER-stress-mediated apoptosis, Bim and PUMA [28], but we did not observe any increased expression of these specific proteins in LPS-induced apoptosis (data not shown).

3.3. LPS administration appears to impair UPR in human hepatoma cells

Next, we focused on the association between TLR4 and UPR in hepatocytes. XBP1 is an important marker of UPR. Two forms of XBP1 have been identified: a spliced form, XBP1(S), and an unspliced form, XBP1(U). Splicing of XBP1 RNA results in the removal of a 26-base intron [26,27,29]. We examined XBP1(S), CHOP, as well as GRP78 mRNA expressions in hepatocytes treated with or without LPS (Fig. 3). In HepG2 cells, we observed that all three gene expression levels were significantly downregulated when cells were treated with 500 ng/mL of LPS (Fig. 3).

We also examined the impairment of UPR by LPS at the protein level in HepG2 cells (Fig. 4). HepG2 cells were treated with LPS or with LPS plus ethanol, and cell lysates prepared after 24 h

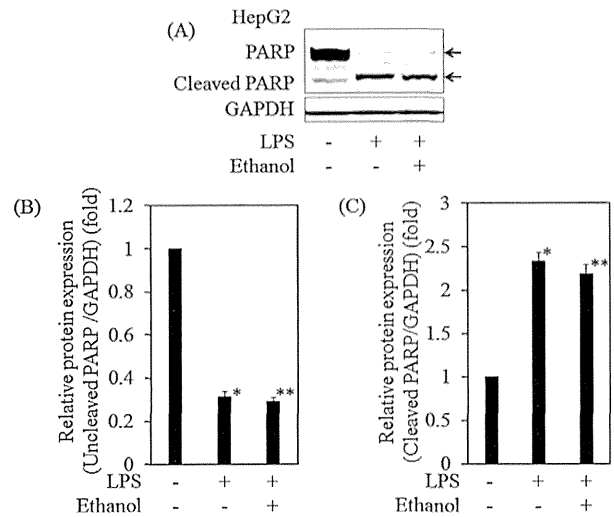

Fig. 2. Lipopolysaccharide (LPS) induced the cleavage of poly(ADP-ribose) polymerase (PARP) in human hepatoma HepG2 cells. (A) Cells were cultured for 24 h with or without TLR4 ligand (LPS, 500 ng/mL) and ethanol (100 mmol/L). Ten micrograms of proteins was subjected to electrophoresis on 7% polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. Western blot analyses of poly(ADP-ribose) polymerase (PARP), and cleaved PARP expression in HepG2 with or without LPS. The uncleaved PARP/GAPDH (B) and cleaved PARP/GAPDH (C) ratios were measured using Scion Image. Data are expressed as mean ± SD of triplicate determinations from 1 experiment representative of 3 independent experiments. (B, $^{*}P = 0.00086$ and $^{**}P = 0.0012$; C, $^{*}P = 0.012$ and $^{**}P = 0.00021$, compared to untreated control by Student's *t*-test.).

of treatment were subjected to Western blot analysis. Downregulation of CHOP, XBP1, as well as their upstream molecule IRE1 was observed in LPS-treated HepG2, compared to untreated HepG2 cells (Fig. 4A–D). There are three pathways in UPR that are mediated by three membrane receptors: PERK, ATF6, and IRE1. We observed the downregulation of ATF4, a downstream molecule of PERK, and ATF6 in LPS-induced HepG2 apoptosis (Fig. 4E–G). We also examined the status of another downstream molecule of PERK, eukaryotic translation initiation factor 2A (eIF2α), and we observed that phosphorylation of eIF2α was also decreased in LPS-treated cells (Fig. 4E, H and I). From these results, LPS administration appears to impair UPR in the human hepatoma cell line HepG2.

3.4. Overexpression of GRP78 rescued LPS-treated HepG2 cells from apoptosis

We also observed the impairment of GRP78 expression at the protein level in HepG2 cells treated with LPS (Fig. 5A and B). Then, we chose to overexpress GRP78 to examine whether this would alter apoptosis in LPS-treated HepG2. We examined apoptosis 1 day after transient transfection of pFLAG/CMV2 or pFLAG-human GRP78 vectors [15] into HepG2 cells and treatment with or without 500 ng/mL of LPS (Fig. 5C–F). After LPS treatment, apoptosis of GRP78-overexpressed HepG2 was reduced compared with that of control ($1.1 \pm 0.58\%$ vs. $14.8 \pm 8.2\%$; $n = 3$, $P = 0.0023$) (Fig. 5E and F). We also observed decreased levels of cleaved PARP in LPS-stimulated HepG2 cells transfected with pFLAG-human GRP78 (pFLAG-hGRP78) compared with those transfected with pFLAG/CMV2 (Fig. 5G and H). These results confirmed that the blocking of GRP78 induction leads to PARP cleavage in hepatocytes.

4. Discussion

Here we report that the downregulation of UPR might be a key step in LPS-induced apoptosis in human hepatoma cells. A number of inherited diseases have been linked to abnormalities in the response to ER stress [30,31]. Several of these cause diabetes, but other diseases associated with ER stress include Parkinson's, familial Alzheimer's and amyotrophic lateral sclerosis. UPR is activated in several liver diseases, including obesity-associated fatty liver disease, viral hepatitis, and alcohol-induced liver injury, all of which are associated with steatosis, raising the possibility that ER stress-dependent alteration in lipid homeostasis is the mechanism underlying steatosis [26,32]. Hepatocyte apoptosis is a pathogenic event in several liver diseases and may be linked to unresolved ER stress [26]. Induction of both ER stress and oxidative stress by hepatitis B virus (HBV) and HCV proteins may also contribute to hepatocyte growth promotion [33,34].

Interestingly, it is already well known that these liver diseases cause HCC. ER stress-mediated cell apoptosis is implicated in the development of cancer [35]. How about the association between ER stress and apoptosis in LPS-stimulated hepatoma cell lines? Our previous study [18] suggested that suppression of anti-apoptotic molecule Bcl-2 expression from mitochondria might play an important role in LPS-treated HepG2. We also observed the activation of caspase-7 and unaltered Bax expression in LPS-induced apoptosis [18], suggesting that GRP78 might also regulate the activation of extrinsic apoptosis as well as that of intrinsic apoptosis

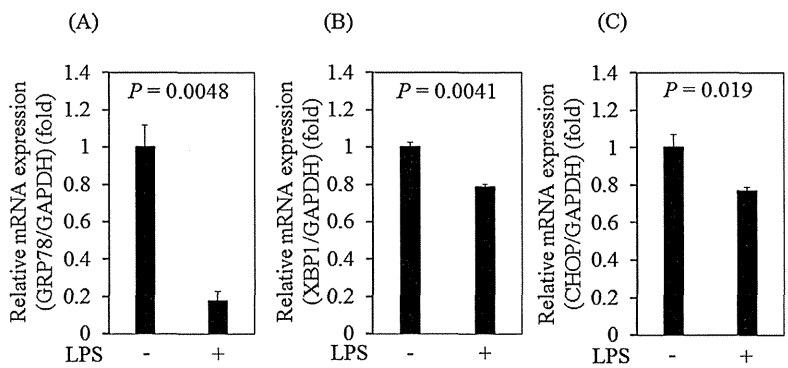

Fig. 3. Effects of lipopolysaccharide (LPS) on unfolded protein response at mRNA levels in HepG2 cells. Cells were cultured for 24 h with 500 ng/mL LPS. Expressions of 78-kDa glucose-regulated protein (GRP78) mRNA (A), X-box binding protein-1 (XBP1) mRNA (B) and C/EBP homologous protein (CHOP) mRNA (C) were examined by real-time RT-PCR in at least triplicates. GAPDH was used for normalization. Data are expressed as mean ± SD of triplicate determinations from 1 experiment representative of 3 independent experiments. *P*-values, compared to untreated control by Student's *t*-test.

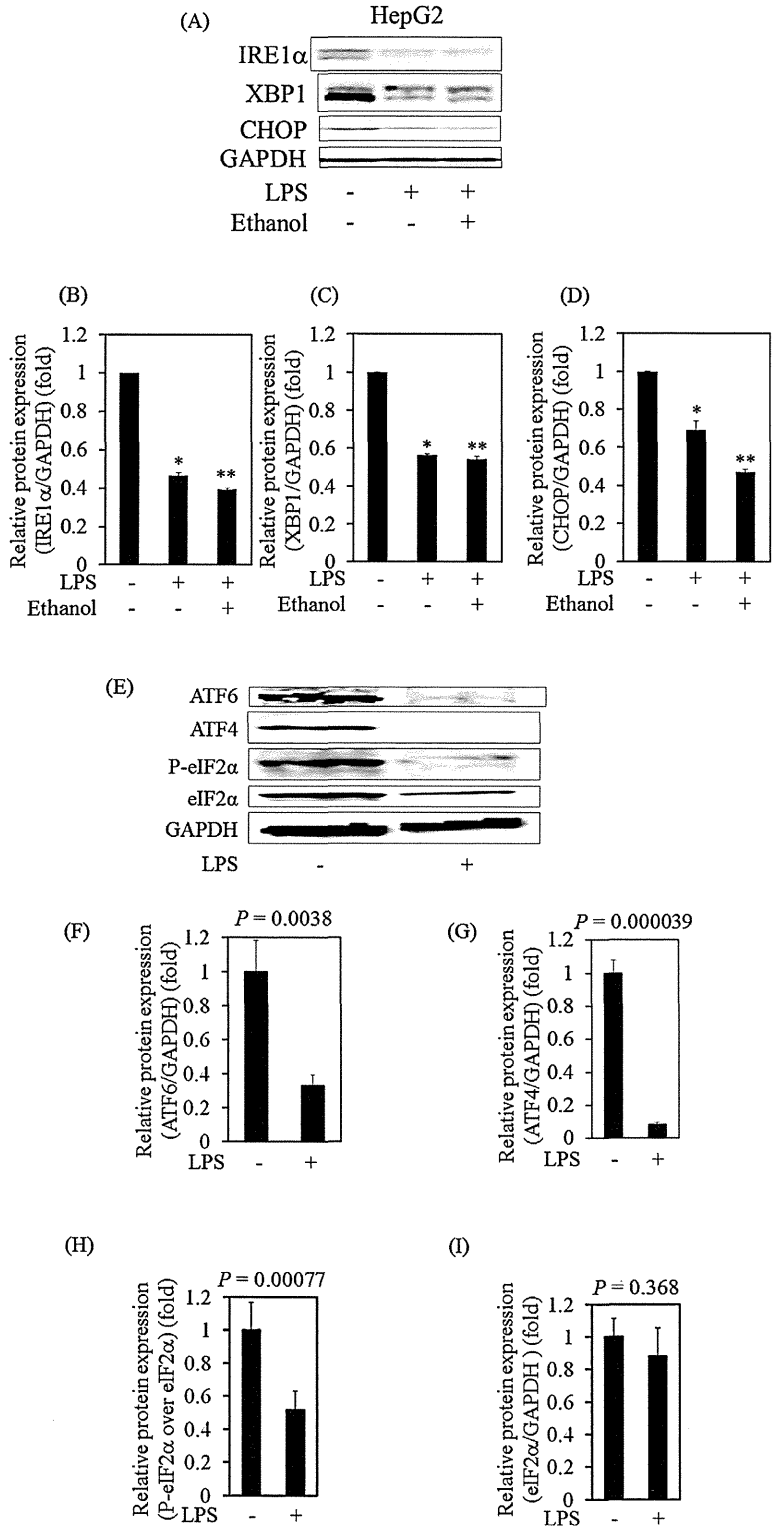

Fig. 4. Effects of lipopolysaccharide (LPS) on unfolded protein response at protein levels in HepG2 cells. (A) Western blot analyses of inositol-requiring enzyme 1α (IRE1α), X-box binding protein-1 (XBP1) and C/EBP homologous protein (CHOP) expression in HepG2 treated for 24 h with or without LPS (500 ng/mL) and ethanol (100 mmol/L). Ten micrograms of proteins was subjected to electrophoresis on 5–20% gradient polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. Blots were re-probed with GAPDH-specific antibodies to assess equal protein loading. IRE1α/GAPDH (B), XBP1/GAPDH (C) and CHOP/GAPDH (D) ratios were measured using Scion Image. (B, $^{*}P = 0.00038$ and $^{**}P = 0.0000080$; C, $^{*}P = 0.00070$ and $^{**}P = 0.00028$; D, $^{*}P = 0.0085$ and $^{**}P = 0.00024$, compared to untreated control by Student's *t*-test.) (E) Western blot analyses of activating transcription factor-6 (ATF6), ATF4, phosphorylation of eukaryotic translation initiation factor 2A (P-eIF2α) and eIF2α expression in HepG2 treated for 24 h with or without LPS (500 ng/mL). Ten micrograms of proteins was subjected to electrophoresis on 5–20% gradient polyacrylamide gels and transferred onto