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Different effects of three interferons L on Toll-like receptor-related gene expression in HepG2 cells

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ABSTRACT

IFNL1 (IL29), IFNL2 (IL28A) and IFNL3 (IL28B) might play important roles in anti-viral defense. IFNL3 genotypes have been shown to be associated with hepatitis C spontaneous and treatment-induced viral clearance.

The effects of IFNL1, IFNL2 and IFNL3 on innate immunity including Toll-like receptor (TLR)-related pathway in human hepatocytes were examined. After G418 screening, we established the human hepatoma stable cell lines HepG2-IL28A, HepG2-IL28B, and HepG2-IL29, expressing IFNL2, IFNL3, and IFNL1 in conditioned medium, respectively, and a control cell line, HepG2-pcDNA3.1. We performed real-time RT-PCR to investigate 84 Toll-like receptor-related gene expressions in triplicate and, using ddCt methods, compared these gene expressions in each cell line.

IFNL2, IFNL3 and IFNL1 were respectively detected by ELISA in HepG2-IL28A, HepG2-IL28B and HepG2-IL29. Compared to HepG2-pcDNA3.1 cells, 17 (20.2%), 11 (13.0%) and 16 genes (19.0%) were up-regulated 1.5-fold or more ($p < 0.05$); 10 (11.9%), 2 (2.3%) and 10 genes (11.9%) were 1.5-fold or more down-regulated ($p < 0.05$) in HepG2-IL28A, HepG2-IL28B and HepG2-IL29, respectively. EIF2AK2 and SARM1 were up-regulated among all cells. Of interest, TLR3, TLR4 and related molecules CXCL10 (IP10), IL6, EIF2K2, IFNB1, and IRF1, important genes in the progression of HCV-related pathogenesis and antiviral activities against HCV, in HepG2-IL28B, presented different profiles from those of HepG2-IL28A and HepG2-IL29.

IFNL3 induces interferon-stimulated genes (ISGs) that are reportedly associated with the progression of HCV-related pathogenesis and antiviral activities against HCV. IFNL is a powerful modulator of innate immune response and it is supposed that the 3 IFNLs may play different roles in the antiviral activity against HBV and HCV.

1. Introduction

Interferons (IFNs)α/β and related molecules are classified as type I IFNs, and two other types have since been identified: type II (IFNγ) and type III (IFNL) [1]. Interleukin-28A (IL28A), IL28B and IL29, also known as IFNL2, IFNL3 and IFNL1, are recognized as type III IFNs [2]. Type I and II IFN receptors are present on most human cell types while type III IFNL receptors are highly expressed on hepatocytes but not on microvascular endothelium, adipocytes, fibroblasts or central nervous system cells [3]. Binding of IFNL1, IFNL2 or IFNL3 to the membrane-associated IFNL receptor (R) complex leads to the activation of the Janus kinases, Janus kinase 1 (Jak1) and tyrosine kinase 2 (Tyk2), subsequent tyrosine phosphorylation of the IFNLR1 intracellular domain (ICD), and activation of the latent transcription factors, signal transducers and activators of transcription 1 (STAT1) and STAT2 [4], recruiting IFN regulatory factor 9 (IRF9) to form the trimeric transcription factor complex IFN-stimulated gene factor 3 (ISGF3), which regulates gene transcription by binding to IFN-stimulated response elements (ISRE) in the promoters of ISGs [5].

Previous comparative cDNA microarray analyses by several groups have shown that the repertoire of genes induced by IFNL is essentially the same as that induced by IFNα/β [5–7]. Exogenous IFNs were used in those studies [6,7]. Disorders of IFNα/β and IFNL production, caused by unc-93 homolog B1 (UNC93B1) and Toll-like receptor 3 (TLR3) mutations, confer a predisposition to herpes simplex encephalitis in otherwise healthy subjects [1,8,9]. IFNL plays a potent role in not only antiviral [10] but also antitumor activities [11]. In host immunity, 5 of the 10 human TLRs (TLR3, TLR4, TLR7, TLR8 and TLR9) can trigger IFNα/β and IFNL, which are critical for antiviral immunity [12]. As the immune system provides the first line of host defenses against microbial pathogens, we chose to study TLR-related genes in the present study.

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IFNL3 genotypes have been shown to be associated with hepatitis C spontaneous and treatment-induced viral clearance [13–18]. IFNL1 is capable of antiviral activity against HCV in vivo [19]. There still exist questions as to whether there might be some differences in the actions among the three IFNLs. We also wondered whether TLR signaling is important for viral eradication through induction of IFNLs. We assumed that IFNLs induced by TLR signaling might further strengthen the antiviral response by inducing TLR-related gene expression, and that this might be important for the positive circuit between the TLR-mediated pathways and IFNLs for the control of viral infection.

In the present study, we made HepG2 cells expressing each of the IFNLs, and the effects of IFNL1, IFNL2 and IFNL3 on innate immunity signaling including the TLR-related pathway in human hepatocytes were examined. Altogether, our data indicate that IFNL is a powerful modulator of innate immune response and suggest that the three IFNLs may play different roles in the antiviral activity against HCV.

2. Materials and methods

2.1. Cells and plasmids

HepG2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) at 37 °C, 5% CO_2. Plasmids pcDNA3.1-IL28A, pcDNA3.1-IL28B and pcDNA3.1-IL29 (kindly provided by Prof. T. Betakova, Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovak Republic [20]) expressed IFNL2, IFNL3 and IFNL1, respectively. Stable HepG2 cell lines expressing IFNL2, IFNL3 or IFNL1 were made as previously described [21]. Briefly, HepG2 cells were transfected with the expression plasmid pcDNA3.1-IL28A, pcDNA3.1-IL28B, pcDNA3.1-IL29 or pcDNA3.1 in Effectene transfection reagent (Qiagen, Hilden, Germany). After 48 h, G418 (Promega, Madison, WI) was added at 1000 μg/mL for selection of HepG2-IL28A, HepG2-IL28B, HepG2-IL29, or HepG2-pcDNA3.1. After 3 weeks, to avoid monoclonal selection, all cells were collected for further analysis.

The HBV DNA (4.1 kbp) construct containing 1.3 copies of HBV genome (subtype *ayw*), kindly provided by Prof. A. McLachlan, University of Illinois at Chicago, IL, USA [22], was used for transient transfection analysis. Plasmid pHCVrep1bBB7 was kindly provided by Prof. C.M. Rice, Washington University School of Medicine, St. Louis, MO, USA [23]. The plasmid contained cDNA coding for an HCV subgenomic replicon.

2.2. RNA extraction

Cells were seeded into 35-mm plates, and total cellular RNA was isolated 48 h later by RNeasy Mini kit (Qiagen) according to the manufacturer's instructions [21]. RNA samples were stored at −80 °C until use. RNA quality was examined using a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. cDNA synthesis, real-time PCR, and PCR array

cDNA synthesis was performed using RT^2 First Strand Kit (Qiagen). Each 1 μg of RNA was subjected to one reaction. cDNA synthesis reaction was as follows: incubation at 42 °C for 15 min and then reaction stoppage by heating at 95 °C for 15 min. Quantitative amplification of cDNA was monitored with SYBR Green by real-time PCR in a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Thermal cycling conditions were 95 °C for 10 min followed by 40 cycles at 95 °C, 15 s for denaturation, and 1 min at 60 °C for annealing and extension. Gene expression profiling of 84 TLR-related genes was performed using RT^2 profiler PCR arrays (Qiagen) following the manufacturer's instructions [24]. Genes were annotated by Entrez Gene (NCBI, Bethesda, MD, USA). 84 genes and functional gene grouping are shown in Table S1.

Gene expression was normalized to two internal controls (GAPDH and β-actin) to determine the fold-change in gene expression between the test sample (HepG2-IL28A/28B/29) and the control sample (HepG2-pcDNA3.1) by 2^{-ddCT} (comparative cycle threshold) method, respectively [24]. Three sets of real-time PCR arrays were performed. Data were analyzed with RT2 Profiler PCR Array Data Analysis software (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php).

2.4. HCV subgenomic RNA electroporated into Huh7 cells and inhibition assay with IFNLs from HepG2-derived cell lines

HCV subgenomic RNA (1 μg) was electroporated into 5×10^6 Huh7 cells using the Gene Pulser Xcell electroporation system (Bio-Rad, Hercules, CA, USA) as described previously [25]. The cells were plated onto 6-well plates. After 48 h, the medium was replaced with conditioned medium from HepG2-pcDNA3.1, HepG2-IL28A, HepG2-IL28B or HepG2-IL29 cells, accompanied by 500 μg/mL G418. Medium was exchanged every 3 days. Two weeks later, the resultant colonies in each well were counted.

2.5. Transfection of HBV DNA construct, HBV DNA extraction and analysis

Cells were seeded onto 35-mm plates. Transfections of HBV DNA construct (0.4 μg/plate) were performed as previously mentioned [21]. Isolation of DNA was performed by SepaGene (Eidia, Tokyo, Japan). DNA samples were stored at −80 °C until use. DNA quality was examined using a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific). Quantitative PCR assay for HBV DNA was performed as previously described [26].

2.6. Enzyme-linked immunosorbent assay (ELISA)

Cell culture fluids were analyzed for human IFNL2, IFNL3 and IFNL1, respectively, using IFNL2 (Abcam Inc., Cambridge, MA, USA), IFNL3 (USCN Life Science Inc., Wuhan, China) and IFNL1 ELISAs (Abnova, Taipei, Taiwan), according to the manufacturers' protocols. The sensitivities of human IFNL2, IFNL3 and IFNL1 by these ELISA kits were 15 pg/mL, 700 pg/mL and 2 pg/mL, respectively.

2.7. Statistical analysis

Student's *t*-test was used to assess statistical significance. Differences with $p < 0.05$ were considered to be statistically significant. Data are expressed as mean ± standard deviations of triplicate determination from 3 independent experiments.

3. Results

3.1. Overexpression of IFNLs in HepG2 cells

It was well known that hepatocytes and the hepatoma-derived cell line HepG2 expressed similar levels of IL28Rα mRNA to the lung epithelial cell line A549, which responds well to IFNLs [4]. We used three protein plasmid vectors under control of the CMV promoter: pcDNA3.1-IL28A, pcDNA3.1-IL28B and pcDNA3.1-IL29 [20,21]. We established three IFN-λs-overexpressing HepG2 cells, designated as HepG2-IL28A, HepG2-IL28B and HepG2-IL29. We also used pcDNA3.1 for the establishment of a control cell, HepG2-pcDNA3.1. To test the ability of these cells to express IFNL2,

IFNL3 and IFNL1, we measured these cytokines by ELISA (Table 1). IFNL2, IFNL3 and IFNL1 could be measured in each cell culture fluid of HepG2-IL28A, HepG2-IL28B and HepG2-IL29, respectively.

3.2. Different IFN-stimulated gene expressions in HepG2 expressing IFNL2, IFNL3 and IFNL1

To gain mechanistic insights into IFNL2, IFNL3 and IFNL1 for ISGs, we used a pathway-specific gene array to identify these IFNLs target genes in HepG2-IL28A, HepG2-IL28B and HepG2-IL29 by comparison with control HepG2-pcDNA3.1. We extracted total RNAs from each of the cells for studying the influence of IFNL2, IFNL3, or IFNL1 overexpression on IFN and cytokine-related genes including TLR signaling pathways using real-time PCR-based array [24]. Compared to HepG2-pcDNA3.1 cells, 17 (20.2%), 11 (13.0%) and 16 genes (19.0%) were up-regulated 1.5-fold or more ($p < 0.05$) (Fig. 1A); 10 (11.9%), 2 (2.3%) and 10 genes (11.9%) were down-regulated 1.5-fold or more ($p < 0.05$) in HepG2-IL28A, HepG2-IL28B and HepG2-IL29, respectively (Fig. 1B). EIF2AK2 and SARM1 were up-regulated in all cells, and PTGS2 and IRAK2 in both HepG2-IL28B and HepG2-IL29, and FOS, IKBKB, IL1A, IRAK1, TLR1, TOLLIP in both IL28A and IL29 ($p < 0.05$) (Fig. 1A). EIF2KA2 is also known as PKR, a serine/threonine protein kinase that is activated by autophosphorylation after binding to dsRNA. EIF2KA2 can mediate the antiviral activity of IFNs, and other IFN-inducible genes can mediate the antiviral effects against HBV and HCV [27,28]. BTK was down-regulated in all cells, CD14 was in both HepG2-IL28B and HepG2-IL29, and CCL2, CD180, CSF2, TLR5 and UBE2N in both HepG2-IL28A and HepG2-IL29 ($p < 0.05$) (Fig. 1B).

3.3. Effects of IFNL2 on IFN and cytokine-related gene expression

Among TLRs and TLR signaling genes, TLR1, TLR8, SARM1, IRAK1, TOLLIP, TIRAP, MAP3K7 and NR2C2 were up-regulated 1.5-fold or more in HepG2-IL28A in comparison to HepG2-pcDNA3.1 ($p < 0.05$, Table 2A) but TICAM2, TLR4, TLR5 and CD180 were down-regulated 1.5-fold or more in HepG2-IL28A ($p < 0.05$, Table 3A). TLR8, which senses single-stranded RNA and plays an important role in the immune response to viral infection, can trigger IFN-α/β and IFNL, which are critical for antiviral immunity [12]. Among pathogen-specific response genes, TLR8, IFNG, EIF2AK, IRAK1, IL10 and RIPK2 were up-regulated 1.5-fold or more in HepG2-IL28A ($p < 0.05$, Table 2A) but CCL2, IFNB1 and CD180 were down-regulated 1.5-fold or more in HepG2-IL28A ($p < 0.05$, Table 3A). Recently, we reported that HBV e antigen physically associates with RIPK2 and regulates IL6 gene expression [29]. Among downstream pathways and target genes, IFNG, IKBKB, IRAK1, IL10, IL1A, MAPK8IP3, FOS and MAP3K7 were up-regulated 1.5-fold or more in HepG2-IL28A ($p < 0.05$, Table 2A) but UBE2N, CSF3, CCL2, IFNB1, CSF2 and BTK were down-regulated 1.5-fold or more in HepG2-IL28A ($p < 0.05$, Table 3A). Among regulation genes with adaptive immunity, IFNG, IL10 and MAP3K7 were up-regulated 1.5-fold or more in HepG2-IL28A ($p < 0.05$, Table 2A). Among adapters and TLR interacting protein genes, SARM1, MAPK8IP3, TOLLIP, TIRAP and RIPK2 were up-regulated 1.5-fold or more in HepG2-IL28A ($p < 0.05$, Table 2A) but TICAM2 and BTK were down-regulated 1.5-fold or more in HepG2-IL28A ($p < 0.05$, Table 3A). Among effector genes, EIF2AK2, IRAK1, MAP3K7 and RIPK2 were up-regulated 1.5-fold or more in HepG2-IL28A ($p < 0.05$, Table 2A) but UBE2N was down-regulated 1.5-fold or more in HepG2-IL28A ($p < 0.05$, Table 3A).

3.4. Effects of IFNL3 on IFN and cytokine-related gene expression

Among TLRs and TLR signaling genes, TLR3, SARM1, IRAK2, MYD88 were up-regulated 1.5-fold or more in HepG2-IL28B, in comparison to HepG2-pcDNA3.1 ($p < 0.05$, Table 2B). Among pathogen-specific response genes, IL6, EIF2AK2, IFNB1, LY96 and PTGS2 were up-regulated 1.5-fold or more in HepG2-IL28B ($p < 0.05$, Table 2B) but CD14 was down-regulated 1.5-fold or more in HepG2-IL28B ($p < 0.05$, Table 3B). Among downstream pathways and target genes, IL6, IFNB1, LY96, IRAK2, IRF1, and NFKBIL1 were up-regulated 1.5-fold or more in HepG2-IL28B ($p < 0.05$, Table 2B) but BTK was down-regulated 1.5-fold or more in HepG2-IL28B

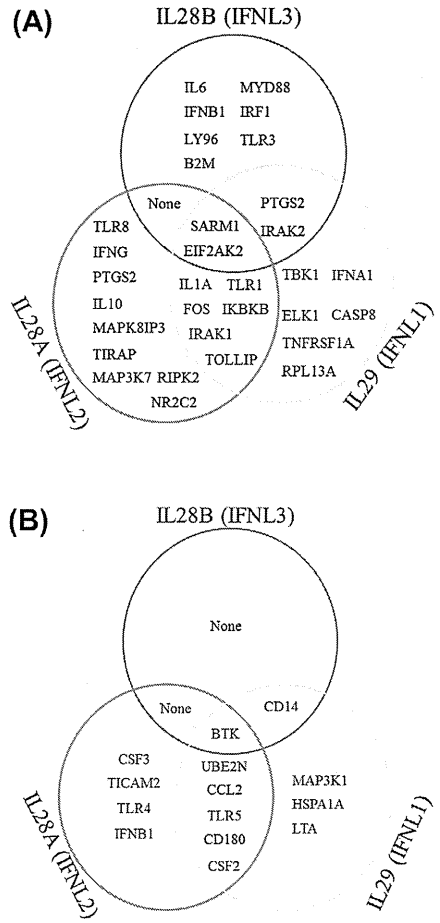

Fig. 1. Venn diagram representing unique and shared Toll-like receptor signaling pathway-related molecules up-regulated or down-regulated by IL28A (IFNL2), IL28B (IFNL3) and IL29 (IFNL1) in human hepatoma cell lines. (A) Toll-like receptor signaling pathway-related genes up-regulated 1.5-fold or more in HepG2-IL28A/HepG2-IL28B/HepG2-IL29 cells compared to HepG2-pcDNA3.1 cells ($p < 0.05$). (B) Toll-like receptor signaling pathway-related genes down-regulated 1.5-fold or more in HepG2-IL28A/HepG2-IL28B/HepG2-IL29 cells compared to HepG2-pcDNA3.1 cells ($p < 0.05$). Three sets of real-time PCR arrays were performed.

Table 1
Overexpressions of IL28A (IFNL2), IL28B (IFNL3), and IL29 (IFNL1) in human hepatoma cell line HepG2. Enzyme-linked immunosorbent assay (ELISA) was performed for IL28A, IL28B or IL29 at least in duplicate.

	HepG2-pcDNA3.1	HepG2-IL28A	HepG2-IL28B	HepG2-IL29
IFNL2 (pg/mL)	<15	4100	<15	<15
IFNL3 (pg/mL)	<700	<700	4100	<700
IFNL1 (pg/mL)	<2	<2	<2	1850

The sensitivities of human IL28A, IL28B and IL29 by these ELISA kits were 15 pg/mL, 700 pg/mL and 2 pg/mL, respectively. 1×10^5 cells were split onto 35-mm plates 48 h before collection of conditioned medium.

Table 2
Up-regulation of Toll-like receptor signaling pathway-related genes in HepG2-IL28A, HepG2-IL28B and HepG2-IL29, compared to HepG2-pcDNA3.1 cells.

Genes	Fold differences	*P*-values
(A) Up-regulated genes (1.5-fold or more) in HepG2-IL28A, compared to HepG2-pcDNA3.1 cells		
TLR10	6.35	0.29
CLEC4E	4.78	0.21
CD80	4.69	0.062
TLR1	3.15	0.00016
TLR8	3.03	0.0086
IFNG	2.87	0.011
CD86	2.22	0.38
EIF2AK2	2.20	0.0016
IKBKB	2.11	0.00093
SARM1	2.02	0.0028
TLR2	2.01	0.12
IRAK1	1.92	0.0011
PTGS2	1.87	0.0043
IL10	1.84	0.038
IL1A	1.76	0.0047
MAPK8IP3	1.75	0.00078
TOLLIP	1.70	0.0091
TIRAP	1.62	0.0099
IL12A	1.58	0.066
FOS	1.57	0.0014
MAP3K7	1.56	0.00091
NR2C2	1.56	0.0013
IFNA1	1.54	0.080
RIPK2	1.52	0.0039
(B) Up-regulated genes (1.5-fold or more) in HepG2-IL28B, compared to HepG2-pcDNA3.1 cells		
TLR3	12.10	0.00031
CXCL10 (IP10)	7.44	0.054
IL6	4.11	0.0012
EIF2AK2	4.06	0.021
IFNB1	3.22	0.00069
LY96	2.96	0.027
B2M	2.38	0.00034
CCL2	2.11	0.088
SARM1	2.06	0.00096
IRAK2	1.83	0.0044
TLR10	1.79	0.25
MYD88	1.71	0.0012
IRF1	1.70	0.0014
NFKBIL1	1.63	0.14
PTGS2	1.60	0.025
TLR4	1.58	0.18
(C) Up-regulated genes (1.5-fold or more) in HepG2-IL29, compared to HepG2-pcDNA3.1 cells		
CLEC4E	5.67	0.23
TLR10	4.05	0.052
TLR8	3.98	0.058
IFNG	3.78	0.062
CD86	2.80	0.20
IL1A	2.67	0.0011
TLR2	2.64	1.02
PTGS2	2.55	0.00017
TLR1	2.51	0.022
IFNA1	2.48	0.019
CD80	2.33	0.053
IKBKB	2.30	0.000012
FOS	2.18	0.000015
TLR3	2.14	0.26
SARM1	1.89	0.0060
IRAK2	1.80	0.019
IRAK1	1.76	0.035
CASP8	1.72	0.0071
IL10	1.70	0.15
EIF2AK2	1.61	0.0084
TBK1	1.60	0.0046
TOLLIP	1.57	0.016
RPL13A	1.56	0.00034
ELK1	1.54	0.0031
TNFRSF1A	1.51	0.027

Table 3
Down-regulation of Toll-like receptor signaling pathway-related genes in HepG2-IL28A, HepG2-IL28B and HepG2-IL29, compared to HepG2-pcDNA3.1 cells.

Genes	Fold differences	*P*-values
(A) Down-regulated genes (1.5-fold or more) in HepG2-IL28A, compared to HepG2-pcDNA3.1 cells		
UBE2N	0.58	0.012
TLR9	0.48	0.85
CSF3	0.45	0.0071
TICAM2	0.41	0.0049
TLR4	0.38	0.00075
CCL2	0.37	0.0015
IFNB1	0.37	0.013
TLR5	0.25	0.0059
CXCL10 (IP10)	0.23	0.10
CD180	0.12	0.0026
CSF2	0.05	0.00081
BTK	0.05	0.0016
(B) Down-regulated genes (1.5-fold or more) in HepG2-IL28B, compared to HepG2-pcDNA3.1 cells		
CSF3	0.66	0.10
CD14	0.65	0.036
CSF2	0.64	0.084
CD86	0.61	0.32
BTK	0.61	0.034
(C) Down-regulated genes (1.5-fold or more) in HepG2-IL29, compared to HepG2-pcDNA3.1 cells		
MAP3K1	0.58	0.0093
UBE2N	0.52	0.0077
CSF3	0.51	0.064
CD14	0.44	0.0033
CXCL10 (IP10)	0.42	0.16
CCL2	0.34	0.0013
HSPA1A	0.34	0.00046
LTA	0.26	0.017
TLR5	0.23	0.0051
CD180	0.12	0.0027
CSF2	0.06	0.00087
BTK	0.04	0.00079

($p < 0.05$, Table 3B). Among adapters and TLR interacting protein genes, LY96, SARM1 and MYD88 were up-regulated 1.5-fold or more in HepG2-IL28B ($p < 0.05$, Table 2B) but CD14 and BTK were down-regulated 1.5-fold or more in HepG2-IL28B ($p < 0.05$, Table 3B). Among effector genes, EIF2AK2 and IRAK2 were up-regulated 1.5-fold or more in HepG2-IL28B ($p < 0.05$, Table 2B). Interestingly, among up-regulated genes (Table 2B), TLR3, TLR4 and related molecules [24,30], CXCL10 (IP10) [31], IL6 [32], EIF2K2 [28], IFNB1 [30], and IRF1 [33], which play important roles in the progression of HCV-related pathogenesis as well as in antiviral activities against HCV, were observed.

3.5. Effects of IFNL1 on IFN and cytokine-related gene expression

Among TLR and TLR signaling genes, TLR1, SARM1, IRAK2, IRAK1, TBK1 and TOLLIP were up-regulated 1.5-fold or more in HepG2-IL29, in comparison to HepG2-pcDNA3.1 ($p < 0.05$, Table 2C), but TLR5 and CD180 were down-regulated 1.5-fold or more in HepG2-IL29 ($p < 0.05$, Table 3C). Among pathogen-specific response genes, PTGS2, FOS, IRAK1, EIF2AK2 and TNFRSF1A were up-regulated 1.5-fold or more in HepG2-IL29 ($p < 0.05$, Table 2C) but CD14, CCL2, HSPA1A and CD180 were down-regulated 1.5-fold or more in HepG2-IL29 ($p < 0.05$, Table 3C). Among downstream pathways and target genes, IL1A, IKBKB, FOS, IRAK2, IRAK1, CASP8, ELK1 and TNFRSF1A were up-regulated 1.5-fold or more in HepG2-IL29 ($p < 0.05$, Table 2C) but MAP3K1, UBE2N, CCL2, CSF2 and BTK were down-regulated 1.5-fold or more in HepG2-IL29 ($p < 0.05$, Table 3C). Among adapters and TLR interacting protein genes, SARM1 and TOLLIP were up-regulated 1.5-fold or more in

HepG2-IL29 ($p < 0.05$, Table 2C) but CD14, HSPA1A and BTK were down-regulated 1.5-fold or more in HepG2-IL29 ($p < 0.05$, Table 3C). Among effector genes, IRAK2, IRAK1, CASP8 and EIF2AK2 were up-regulated 1.5-fold or more in HepG2-IL29 ($p < 0.05$, Table 2C) but UBE2N was down-regulated 1.5-fold or more in HepG2-IL29 ($p < 0.05$, Table 3C).

3.6. Antiviral effects of IFNLs on hepatitis viruses

We previously reported that IFNL1 inhibits HCV JFH1 replication in human hepatoma cell Huh7-derived cell lines [21]. Because it seemed that HCV could not replicate in HepG2 cells, we examined whether IFNLs produced from HepG2-derived cell lines could inhibit HCV subgenomic RNA replication in Huh7 cells using colony formation assay (Fig. 2A). Although 8.3 ± 2.0 colonies/field (magnification 40×) were identified in Huh7 cells treated with conditioned medium from HepG2-pcDNA3.1, we did not observe any G418-resistant colonies in Huh7 cells treated with that from HepG2-IL28A, HepG2-IL28B or HepG2-IL29 for 2 weeks, suggesting that the 3 kinds of IFNLs could inhibit HCV subgenomic RNA replication.

Next, we determined whether IFNLs could inhibit HBV replication after HBV transfection. For this, we transfected pCMV-HBV DNA into HepG2-pcDNA3.1, HepG2-IL28A, HepG2-IL28B and HepG2-IL29 cells. Cells were incubated for 10 h after transfection, washed three times, and then incubated with fresh medium. Medium was exchanged at 24, 48, 72 and 96 h after transfection. Cellular DNA was collected at 96 h after transfection and HBV DNA was tested by real-time PCR. Conditioned medium was also collected for HBV DNA quantitation at 24, 48, 72 and 96 h after transfection. We obtained 14.3% ($n = 3$, $p < 0.05$), 56.2% ($n = 3$, $p < 0.05$) and 80.2% ($n = 3$, $p < 0.05$) of intracellular HBV DNA levels in HepG2-IL28A, HepG2-IL28B and HepG2-IL29, respectively, compared to 100% ($n = 3$) in HepG2-pcDNA3.1 (Fig. 2B). In conditioned medium at 96 h after transfection, HBV DNA levels were 8.6, 7.8, 7.9 and 7.8 log copies/mL in HepG2-pcDNA3.1, HepG2-IL28A, HepG2-IL28B and HepG2-IL29, respectively, although we did not observe any differences in HBV DNA levels at 24, 48 and 72 h after transfection.

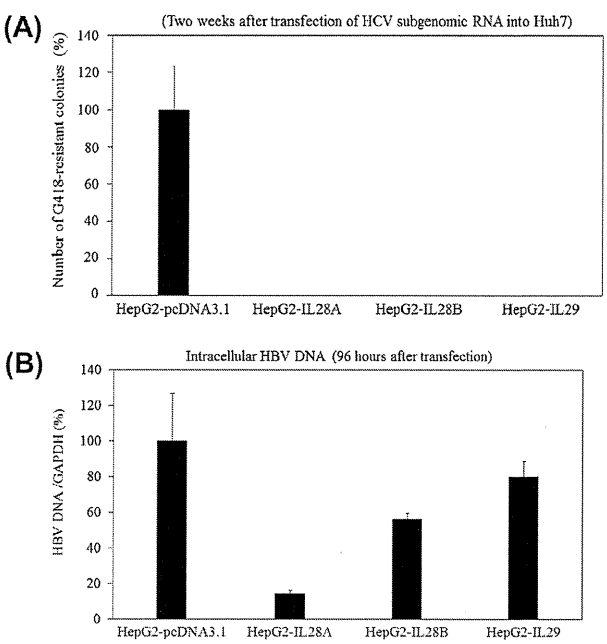

Fig. 2. IFNLs produced from HepG2-derived cell lines could inhibit HCV and HBV replication in human hepatoma cell lines. (A) IFNLs produced from HepG2-derived cell lines could inhibit HCV subgenomic RNA replication in Huh7 cells. HCV subgenomic RNA were electroporated into Huh7 cells [25]. Two weeks after incubation with condition medium from HepG2-pcDNA3.1, HepG2-IL28A, HepG2-IL28B, HepG2-IL29, accompanied with 500 μg/mL G418, the resultant colonies were counted. The number of G418-resistant colonies in the well treated with condition medium from HepG2-pcDNA3.1 was set as 100%. Data are expressed as mean ± SD. (B) HBV replication was inhibited in HepG2-IL28A, HepG2-IL28B, HepG2-IL29, compared with HepG2-pcDNA3.1. We transfected pCMV-HBV DNA (0.4 μg) into HepG2-pcDNA3.1, HepG2-IL28A, HepG2-IL28B and HepG2-IL29 cells. Cellular DNA was collected at 96 h after transfection and HBV DNA was examined by real-time PCR [26]. HBV DNA/GAPDH levels from HepG2-pcDNA3.1 were set as 1. Data are expressed as mean ± SD.

4. Discussion

In the present study, we showed that three different IFNLs induced expressions of TLR-related genes in human hepatocytes. Our results also showed that IFNL3 induced TLR signaling-related genes that play key roles in the elimination of HCV (Table 2B), supporting the results from recent genome-wide association studies identifying the association between IFNL3 genotypes and natural or treatment-induced HCV elimination [13,14,34,35]. We demonstrated that IFNLs produced by HepG2-derived cell lines could inhibit HBV and HCV replication, supporting the previous study [21].

It has been reported that TLR agonists induce a different expression of type I IFNs and IFNLs [36]. TLR3-, TLR7-, TLR8-, and TLR9-mediated inductions of IFNαβ and IFNL were reported to be important for controlling viral infection [37–41]. Wang et al. [41] reported that TLR3 agonists induce IFNLs and that IFNLs play an important role in anti-HCV activity in hepatic stellate cells (HSCs).

In the present study, we focused on hepatocytes, where HBV and HCV can replicate and which express IFNL receptors. Human HSCs [41] and plasmacytoid dendritic cells [42] can produce IFNLs. Recently, Yoshio et al. [43] reported that human blood dendritic cell antigen 3 $(BDCA3)^+$ dendritic cells prodigiously produce IFNL in response to HCV. Are TLR signalings important for viral clearance through induction of IFNLs? We assumed that IFNLs induced by TLR signaling might further strengthen the antiviral response by inducing the expressions of TLR-related genes. That is, we hypothesized the importance of the positive circuit between the TRL-mediated pathways. To examine whether HepG2 cells overexpressing IFNLs respond more strongly to the corresponding TLR agonists than do control HepG2-pcDNA3.1 cells, we examined IFN-β promoter activities after poly(I-C) transfection into HepG2-IL28B cells [30], which enhanced TLR3 expression (Table 2B). Unexpectedly, however, we did not observe any enhancement of IFN-β promoter activities in HepG2-IL28B, in comparison with HepG2-pcDNA3.1 (data not shown). Further studies will be needed to clarify the roles of IFNLs for viral eradication.

There exist two type I IFNs, IFN-α and IFN-β, which use the same type I IFN receptor. But the clinical effects are a little different between these two type I IFNs. For example, IFN-α and IFN-β are now the first lines of treatment for chronic hepatitis C infection and for relapsing remitting multiple sclerosis, respectively [3]. There are three type III IFNs, IFNL1, IFNL2 and IFNL3, which might use the same IFNL receptor. The present study also indicated that the actions are different among these three IFNLs on ISG expression, although a limitation of our system concerning the dose of each IFN might exist (Table 1). Future studies will be needed to clarify this point and the underlying mechanisms. Kohli et al. [44] reported that the majority of genes induced by IFNα and IFNL were similar, IFNL exhibited profound, but delayed kinetics of ISGs induction while IFNα induced ISGs more rapidly, and that there were different activities of IFN-α and IFNL between HCV-infected and non-infected cells [44]. Although all three IFNLs induced EIF2K2 and SARM1 (Fig. 1A), genes induced by IFNL3 seemed to be different from those by IFNL2 or IFNL1. It was reported that

SARM1 was a negative regulator of innate immunity in neuronal morphogenesis [45].

Retinoic acid inducible-gene I (RIG-I)-like receptors (RLRs) are other important innate immune gatekeepers involved in viral clearance. RIG-I is one of the sensors of HCV RNA and plays an important role in anti-HCV defenses [46]. In HCV infection, HCV pathogen-associated molecular pattern (PAMP) triggers production of IFNLs through RIG-I [42]. RLRs, as well as TLRs, might be important in the production of IFNLs [47,48]. Further studies will be needed to determine whether IFNLs are involved in the RLR-mediated signal pathways in antiviral responses against HBV, although we have reported that HBeAg could interact with receptor-interacting serine/threonine protein kinase-2 (RIP2/RIPK2) [29].

Our results supported the previous studies [6,7,19,21,49] showing that IFNL could suppress HCV and HBV replication. These findings and our observation suggest that the combination of these IFNLs might be useful for the control of chronic hepatitis C and B, as well as other viral infections. Recent studies suggested that a novel IFNL4 also plays an important role in the host response to HCV [50,51]. In conclusion, three types of IFNLs induced different expressions of TLR-related genes, suggesting that they play different roles in liver diseases.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cyto.2013.08.010.

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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

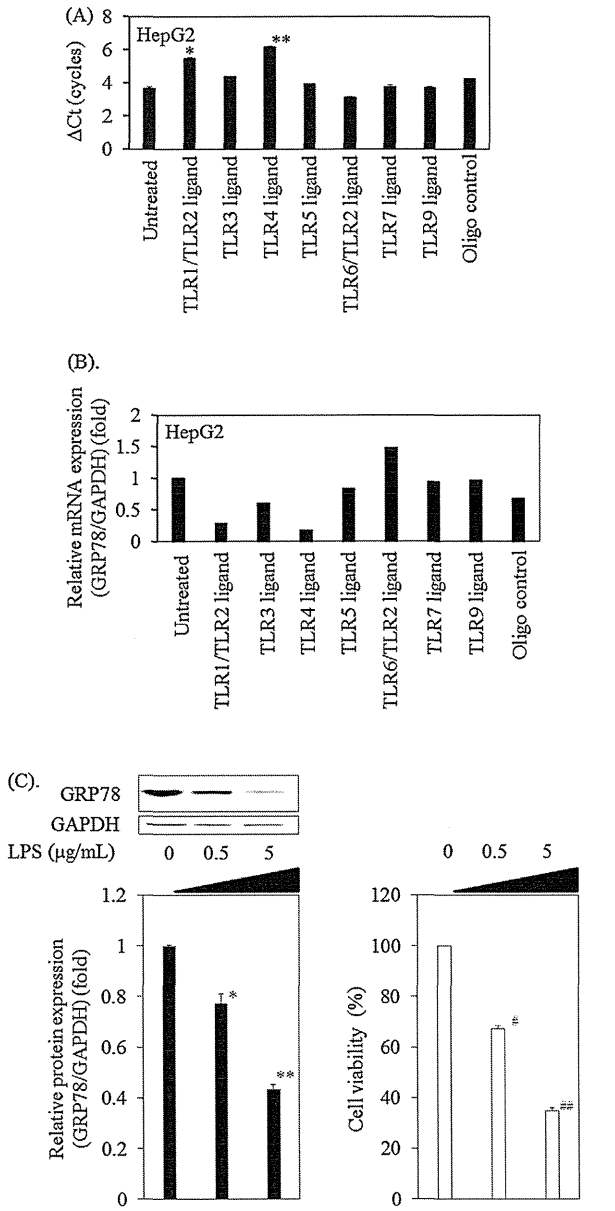

Fig. 1. Effects of TLR ligands on 78-kDa glucose-regulated protein (GRP78) mRNA expression in human hepatoma HepG2 cells. HepG2 cells (A, B) were cultured for 24 h with ligands of Toll-like receptor (TLR)1/TLR2 (Pam3CSK4), TLR3 (Poly[I–C]), TLR4 (LPS), TLR5 (Flagellin), TLR6/TLR2 (MALP-2), TLR7 (Imiquimod [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

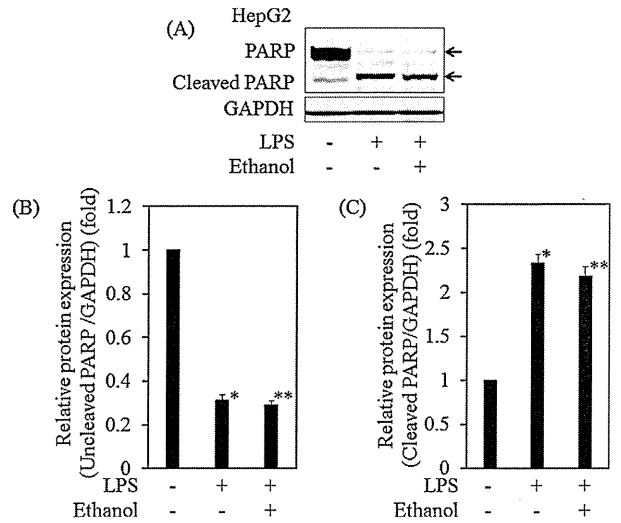

Fig. 2. Lipopolysaccharide (LPS) induced the cleavage of poly(ADP-ribose) polymerase (PARP) in human hepatoma HepG2 cells. (A) Cells were cultured for 24 h with or without TLR4 ligand (LPS, 500 ng/mL) and ethanol (100 mmol/L). Ten micrograms of proteins was subjected to electrophoresis on 7% polyacrylamide 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

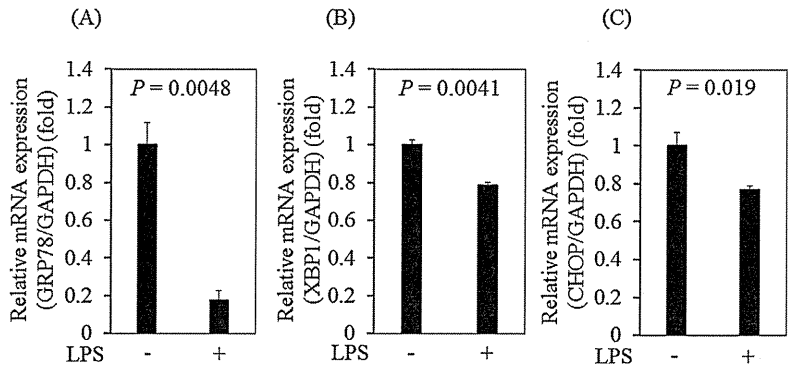

Fig. 3. Effects of lipopolysaccharide (LPS) on unfolded protein response at mRNA levels in HepG2 cells. Cells were cultured for 24 h with 500 ng/mL LPS. Expressions of 78-kDa glucose-regulated protein (GRP78) mRNA (A), X-box binding protein-1 (XBP1) mRNA (B) and C/EBP homologous protein (CHOP) mRNA (C) 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.

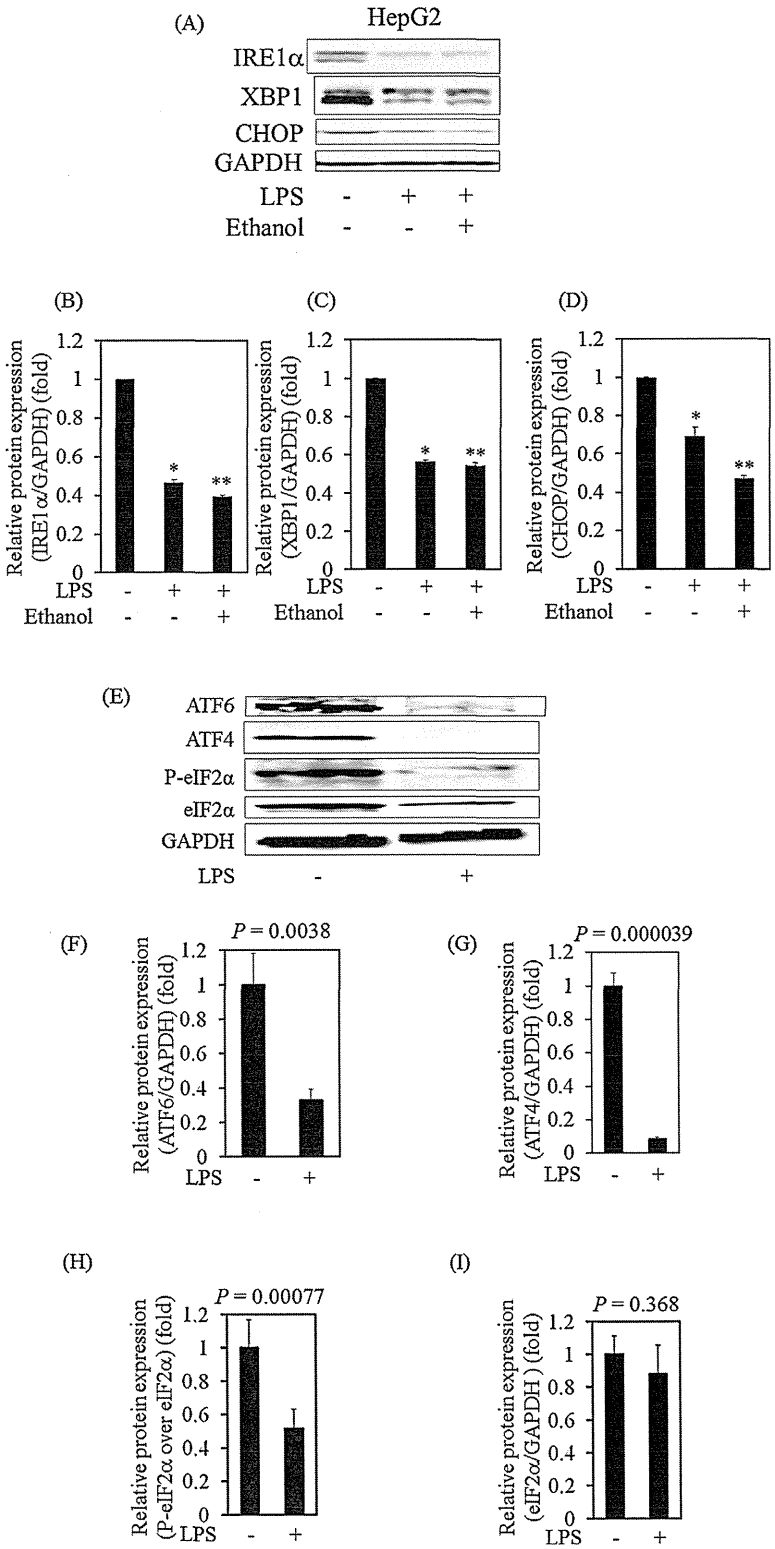

Fig. 4. Effects of lipopolysaccharide (LPS) on unfolded protein response at protein levels in HepG2 cells. (A) Western blot analyses of inositol-requiring enzyme 1α (IRE1α), X-box binding protein-1 (XBP1) and C/EBP homologous protein (CHOP) expression in HepG2 treated for 24 h with or without LPS (500 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

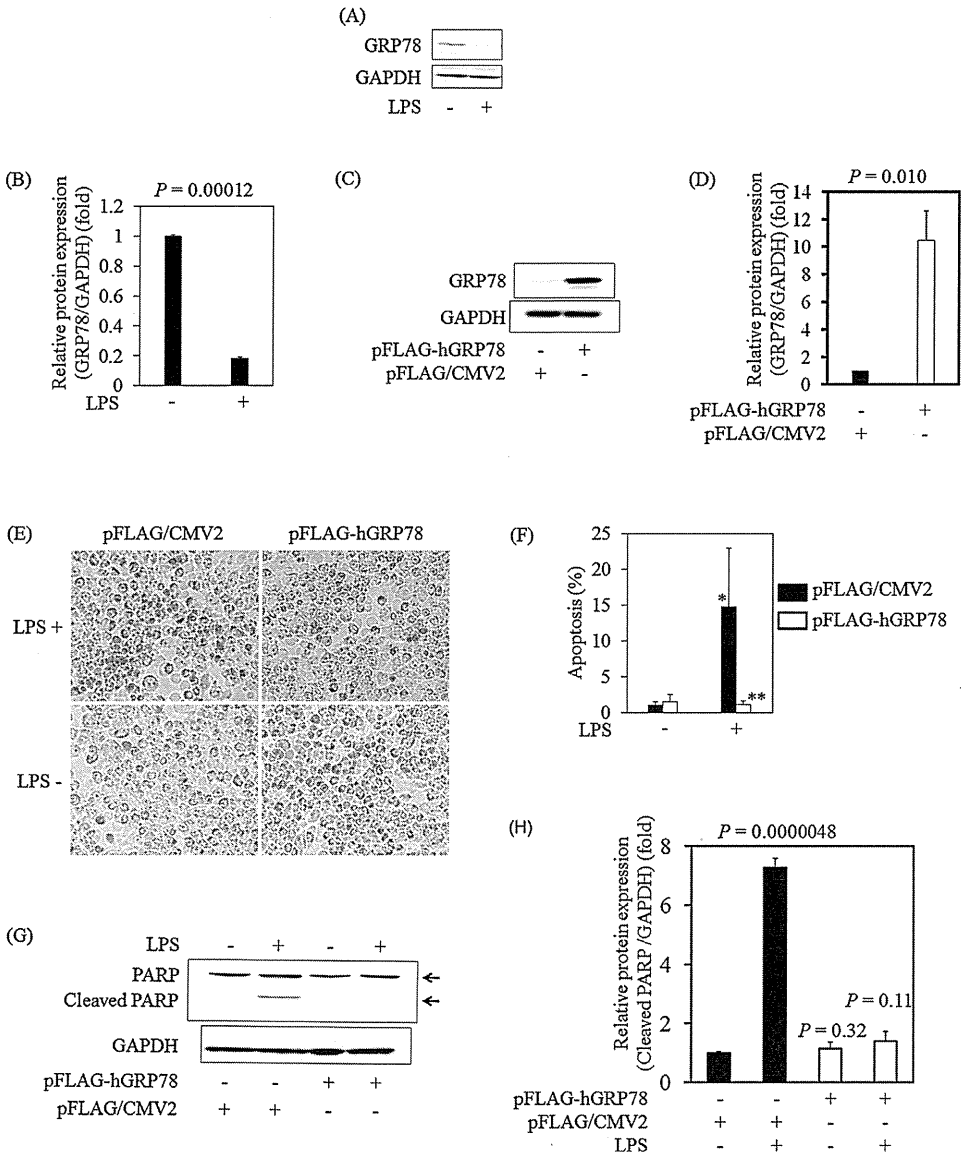

Fig. 5. Overexpression of GRP78 rescued LPS-treated HepG2 cells from cell death. (A) Western blot analysis of GRP78 expression in HepG2 treated for 24 h with or without LPS (500 ng/mL). Four micrograms of proteins was subjected to electrophoresis on 5–20% gradient polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. Blots were re-probed with GAPDH-specific antibodies to assess equal protein loading. (B) GRP78/GAPDH ratios were measured using Scion Image. (C) Overexpression of human GRP78 protein separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by Western blot. Cells were transfected with 0.4 μg pFLAG-human (h) GRP78 or pFLAG/CMV2 control vectors [15] and 2 days later, cellular proteins were collected with 1× SDS lysis buffer. Four micrograms of proteins was subjected to electrophoresis on 5–20% gradient polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. (D) GRP78/GAPDH ratios were measured using Scion Image. (E, F) Cell apoptosis was quantified by APOPercentage Apoptosis Assay. HepG2 cells transfected with 0.2 μg pFLAG/CMV2 control vectors (black column) or 0.2 μg pFLAG-hGRP78 (white column) were cultured for 24 h with or without LPS (500 ng/mL). 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]. *$P = 0.043$, compared to untreated control by Student's t-test. **$P = 0.044$, between HepG2 transfected with pFLAG-hGRP78 and with control vectors. (G) Overexpression of GRP78 reduced LPS-stimulated poly(ADP-ribose) polymerase (PARP) cleavage in HepG2. Western blot analyses of poly(adenosine diphosphate-ribose) polymerase (PARP) expression in pFLAG/CMV2 or pFLAG-hGRP78-transfected cells treated for 24 h with LPS. Four micrograms of proteins was subjected to electrophoresis on 7% polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. Blots were reprobed with GAPDH-specific antibodies to assess equal protein loading. (H) Densitometric analysis of cleaved PARP/GAPDH showing increased cleaved PARP after transfection with pFLAG/CMV2 and LPS-treatment in HepG2 cells. Transfection of pFLAG-hGRP78 blocked LPS-induced PARP cleavage. Cleaved PARP/GAPDH ratios were measured using Scion Image. Data from three independent experiments were measured using Scion Image. Data are expressed as mean ± SD.

polyvinylidene difluoride membranes. Blots were re-probed with GAPDH-specific antibodies to assess equal protein loading. ATF6/GAPDH (F), ATF4/GAPDH (G), P-eIF2α/total eIF2α (H) and eIF2α/GAPDH (I) ratios were measured using Scion Image. Blots were re-probed with GAPDH-specific antibodies to assess equal protein loading. Results are expressed as mean ± SD of triplicate determinations from 1 experiment representative of 3 independent experiments.

pathways. It was recently reported that the commitment phase of ER stress-induced apoptosis is largely dependent on mitochondria [7]. In the present study, we did not observe the induction of CHOP by LPS in HepG2 (Fig. 3C), which is reported to cause apoptosis, although we observed LPS-induced apoptosis in HepG2. Although Zha et al. [35] reported that tunicamycin induced HepG2 apoptosis concomitantly with the upregulation of pro-apoptotic transcription factor CHOP and downregulation of Bcl-2, CHOP might not be essential in LPS-induced hepatic apoptosis. But we did not observe the enhancement of hepatic apoptosis with LPS treatment with ER stress-induced media including thapsigargin (data not shown). Further studies focusing on the relationship between CHOP and hepatic apoptosis will be needed. Recent evidence indicates that the cell homeostasis program triggered by ER stress intersects with the innate immune response [36]. Our results support the previous report that the ER stress response might be part of the conserved innate immune recognition system [37].

Previous studies [33,34] reported that HBV and HCV infections induced ER stress and oxidative stress in the liver. HBV and HCV infections induce ER stress, perhaps as a result of a weakening innate immune response including the TLR signaling pathway [17,38]. Further studies will be needed to clarify this. In the present study, we observed that UPR was reduced when LPS induced apoptosis in human hepatoma cell lines. Innate immune response seems to be closely associated with UPR. It was reported that GRP78 promotes tumor proliferation, survival, metastasis and resistance to a wide variety of therapies [9] and that GRP78 is upregulated in HCC [20]. In the present study, we also observed that the overexpression of GRP78 prevented hepatoma cells from reaching apoptosis, suggesting that GRP78 may play an important role in the resistance against therapies for HCC.

It was also reported that UPR inhibits cisplatin-induced apoptosis in HCC cells [39]. Shi et al. [40] reported that targeting autophagy enhances sorafenib lethality for HCC via ER stress-related apoptosis. The combination of ER stress-associated cell death and molecular-targeted therapy might represent a promising therapeutic strategy for the treatment of HCC. In conclusion, when LPS induced apoptosis in human hepatoma cell lines, we observed that UPR was impaired in these same cell lines. Innate immune response including TLR signaling seems to play an important role in UPR. Of interest, the interaction of inflammation and ER stress seems to control hepatoma apoptosis, presenting compelling clinical implications. Our study showed that UPR downregulation could be a collateral effect of LPS treatment, suggesting that UPR determines hepatic cell damage induced by an innate immune response including TLR signaling pathways. Still, little is known about the detailed molecular mechanisms that allow them to play different roles in hepatic cell apoptosis.

Disclosure statement

No competing financial interests exist.

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Prevalence of Hepatitis C Virus Subgenotypes 1a and 1b in Japanese Patients: Ultra-Deep Sequencing Analysis of HCV NS5B Genotype-Specific Region

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Abstract

Background: Hepatitis C virus (HCV) subgenotypes 1a and 1b have different impacts on the treatment response to peginterferon plus ribavirin with direct-acting antivirals (DAAs) against patients infected with HCV genotype 1, as the emergence rates of resistance mutations are different between these two subgenotypes. In Japan, almost all of HCV genotype 1 belongs to subgenotype 1b.

Methods and Findings: To determine HCV subgenotype 1a or 1b in Japanese patients infected with HCV genotype 1, real-time PCR-based method and Sanger method were used for the HCV NS5B region. HCV subgenotypes were determined in 90% by real-time PCR-based method. We also analyzed the specific probe regions for HCV subgenotypes 1a and 1b using ultra-deep sequencing, and uncovered mutations that could not be revealed using direct-sequencing by Sanger method. We estimated the prevalence of HCV subgenotype 1a as 1.2-2.5% of HCV genotype 1 patients in Japan.

Conclusions: Although real-time PCR-based HCV subgenotyping method seems fair for differentiating HCV subgenotypes 1a and 1b, it may not be sufficient for clinical practice. Ultra-deep sequencing is useful for revealing the resistant strain(s) of HCV before DAA treatment as well as mixed infection with different genotypes or subgenotypes of HCV.

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Introduction

Hepatitis C virus (HCV) infection causes acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma [1,2]. Worldwide, approximately 170 million people are chronically infected with HCV [3]. HCV is a single-stranded RNA virus ~9600 nt in size, and it belongs to the *Flaviviridae family*. HCV genomes are translated into a single open reading frame of ~3000 amino acids, and by cellular and viral encoded-protease are processed into structural (core, E1, E2 and p7) and non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) [4]. HCV is classified into six major genotypes and there are nucleotide differences between HCV genotypes at more than 30%. HCV subgenotypes such as 1a and 1b in HCV genotype 1 also exist, and they are typically different from each other at 20% to 25% of nucleotides [5].

Standard of care (SOC) for chronic hepatitis C is based on a combination of pegylated-interferon and ribavirin for 48 weeks, which leads to only ~50% sustained virological response (SVR) in patients with HCV genotype 1. Contrary to HCV genotype 1, SOC for 24 weeks leads to 70~80% SVR in HCV genotype 2- or 3-infected individuals [4]. Thus, there are differences in treatment response among HCV genotypes.

Recently, two direct-acting antivirals (DAAs) against HCV, telaprevir and boceprevir, were introduced, and they are now available in combination with peginterferon plus ribavirin for

treatment of chronic hepatitis C [6]. The addition of these protease inhibitors to SOC leads to significantly higher SVR rates than SOC only in previous-treatment relapsers and untreated patients infected with HCV genotype 1 [6]. It is well known that telaprevir- and boceprevir-based therapies are relatively more effective in HCV subgenotype 1b patients than in those with HCV subgenotype 1a [7,8]. Although we expect to be using other DAAs and interferon-free regimens for therapies of chronic hepatitis C in the near future, the treatment response rate might be low in HCV subgenotype 1a compared to HCV subgenotype 1b infection [9,10]. Thus, HCV genotype or subgenotype is one of the predictors of the response to anti-viral therapy.

In Japan, the proportions of HCV genotypes 1 and 2 are 70% and 30%, respectively. The prevalence of HCV subgenotype 1a in HCV genotype 1, after excluding patients with hemophilia, was reported to be ~1% [11]. However, it is possible that non-responder patients might be associated with HCV subgenotype 1a in Japan. Therefore, it seems important to distinguish between HCV subgenotypes 1a and 1b in Japan as well as in other countries.

To reveal the current prevalence of HCV subgenotype 1a in Japanese HCV genotype 1-infected individuals, we examined HCV subgenotypes 1a and 1b. We also examined the method for identifying HCV genotypes by real-time PCR-based method in the HCV NS5B region [12] and analyzed the specific probe region for HCV subgenotypes 1a and 1b using ultra-deep sequencing.

Results

Subgenotyping by one-step real-time PCR with MGB probe in the HCV NS5B region

In the present study, HCV genotypes of all samples were determined by the antibody serotyping method of Tukiyama-Kohara et al. [13,14]. According to this assay, HCV serotypes 1 and 2 correspond to HCV genotypes 1a/1b and 2a/2b [5]. The clinical background of 80 patients was shown in Table 1. All but 2 patients had high viral loads. First, all 80 patients were Japanese, and their HCV subgenotypes were determined by real-time PCR-based method with MGB probe in the HCV NS5B region [12]. HCV subgenotypes were determined in 74 of the 80 patients: 4 (5.0%) and 70 (87.5%) were classified into HCV subgenotypes 1a and 1b, respectively. HCV subgenotypes remained undetermined by this method in the other 6 patients (7.5%).

Comparison of the result from HCV subgenotyping by real-time PCR-based method in the HCV NS5B region and that from direct-sequencing by Sanger method in Japanese patients infected with HCV genotype 1

It has been reported that the prevalence of HCV subgenotype 1a was ~1% in Japan [11]. Our result from the real-time PCR-based subgenotyping method suggested that this prevalence might be a little higher although our study population of 80 patients was too small to be considered representative. We tried to perform direct-sequencing by the Sanger method in 10 patients: 4 with HCV subgenotype 1a and 6 with undetermined HCV subgenotypes by real-time PCR-based method [12]. Because the PCR product in 1 of the 4 HCV subgenotype 1a patients could not be amplified, this patient was excluded (Table 2, patient No. 69).

TaqMan MGB probes exhibit great differences in Tm values between matched and mismatched probes, providing more accurate allelic discrimination and making for a more sensitive real-time assay [12]. Thus, we then performed direct-sequencing by the Sanger method in 9 patients. As shown in Table 2, only 1 of three patients classified into HCV subgenotype 1a by real-time PCR-based method [12] was confirmed to be HCV subgenotype 1a (Table 2, patient No. 74). All other samples with subgenotype 1a or undetermined by real-time PCR-based method, were confirmed to be HCV subgenotype 1b by direct-sequencing (Table 2). In accordance with the alignments of the MGB probe segments from each patient in comparison to those in H77 and Con1, we found that patients No. 46 and 48 showed an A to G mutation at

Table 1. Clinical characteristics of HCV genotype 1-infected in the present study.

Number of patients (male/female)	80 (39/41)
Age (years)	51±14
HCV RNA levels (low/high)	2/78
ALT (IU/L)	67±41
WBC (x10^3/μL)	5.5±1.7
Hemoglobin (g/dL)	14±1.1
Platelet counts (x10^4/μL)	18±5.8
γ-GTP (IU/L)	53±70
IL28B rs8099917, TT/TG/GG	49/30/1

Note: HCV RNA levels, low: less than 5 log IU/mL; HCV RNA levels, high: equal to and more than 5 log IU/mL; ALT, alanine aminotransferase; WBC, white blood cell count.

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Table 2. Results of HCV subgenotype determining by real-time PCR-based method and direct-sequencing by Sanger method.

Patient No.	Real-time PCR-based subgenotyping method	Direct-sequencing by Sanger method
4	undetermined	1b
25	undetermined	1b
37	undetermined	1b
46	1a	1b
47	undetermined	1b
48	1a	1b
66	undetermined	1b
69*	1a	undetermined*
74	1a	1a
78	undetermined	1b

Note: * The PCR product of patient No. 69 could not be obtained before performing direct-sequencing.

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Table 3. Nucleotide sequences in MGB probe segments of each patient.

Refs.	H77 from 8913 to 8926 CAGCTTGAACAGGC	Con1 from 8910 to 8923 CAACTTGAAAAAGC
No. 4	---------A----	--G-------G--
No. 25	--A------A-A--	----------
No. 37	--A------A-A--	----------
No. 46	-------G-A-A--	--G----G------
No. 47	--A------A-A--	----------
No. 48	--A-----GA-A--	--------G-----
No. 66	--A------A-A--	----------
No. 74	----------	--G------C-G--
No. 78	--A------A-A--	----------

Reference sequences (Refs) were obtained from HCV subgenotype 1a (H77) and HCV subgenotype 1b (Con1). GenBank accession No.: H77, AF009606.1; Con1, AJ238799.1.

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Fig. 1

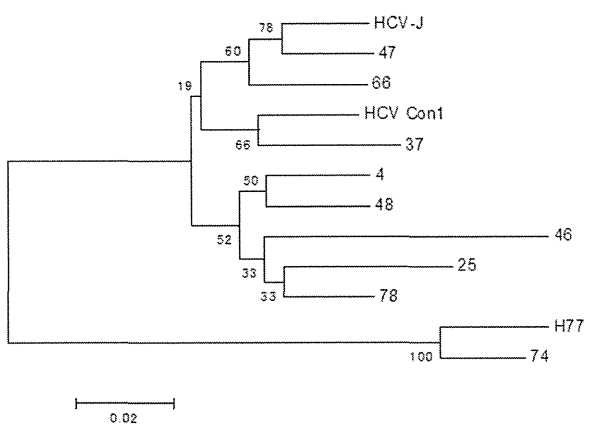

Figure 1. Phylogenetic tree analysis by the Neighbor-Joining (NJ) method. The numbers at the branches are confidence values based on Felsenstein's bootstrap analysis (500 replicates) with MEGA version 4 [28]. HCV HCV-J, HCV Con1 and HCV H77 strains belong to HCV subgenotypes 1b, 1b and 1a, respectively. GenBank accession No: HCV-J, D90208.1; Con1, AJ238799.1; H77, AF009606.1.

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nucleotide site 8917 and 8918 (reference to Con1), respectively (Table 3).

Phylogenetic tree analysis. We also constructed a phylogenetic tree based on the 9 patients' sequences of about 424 bp length containing the HCV NS5B probe region obtained using direct-sequencing by the Sanger method. The phylogenetic tree showed that only one patient, No. 74, belonged to HCV subgenotype 1a, while the others all clustered to HCV subgenotype 1b (Figure 1).

Calculation of PCR and Roche/454 GS Junior sequencer error rates. In order to ensure that errors introduced by PCR as well as errors inherent to the Roche/454 pyrosequencing technology were below our minimum variant frequency threshold of 1%, we sequenced the PCR products from 10^3 to 10^4 copies of control plasmid and found no mutations, indicating similar error rates lower than 1%.

Sequencing read lengths of No. 4, 25, 37, 46, 47, 48, 64, 74 and 78 were 5151, 3725, 6075, 5145, 5071, 3939, 8990, 4002 and 9227, respectively. The coverage numbers were 2078-9195 at each position.

Performance comparison of ultra-deep sequencing and Sanger direct-sequencing. To explore the difference between ultra-deep sequencing and direct-sequencing by the Sanger method, we compared the differences in nucleotide sequences between the two methods (Tables 3 4). Direct-sequencing showed that only 3 of 8 HCV subgenotype 1b isolates (37.5%) had nucleotide sequence variations, compared to MGB probe sequence, according to HCV Con1. Concerning HCV subgenotype 1a isolate, direct-sequencing showed no nucleotide sequence variations, compared to MGB probe sequence, according to HCV H77.

However, analysis of ultra-deep sequences showed that 6 of 8 HCV subgenotype 1b isolates (75%) had nucleotide sequence variations (1%), compared to MGB probe sequence, according to HCV Con1. In No. 37 and No. 47, respectively, one mutation [8912:A/G mutation (27%)] and four mutations [8913.5:/G (30%), 8915.5:/A (70%), 8917:A/G (30%) and 8921.5:/G (70%)] were identified in ultra-deep sequences. Similarly, in No. 78, one mutation [8922.5:/G (1.09%)] was identified (Table 4).

Even in the HCV subgenotype 1a isolate, analysis of ultra-deep sequences showed one nucleotide sequence variation (1%), compared to MGB probe sequence, according to HCV H77 (Table 4). We also compared nucleotide sequence variations between MGB probe and non-MGB probe regions (data not shown). In 8 HCV subgenotype 1 patients, we found no difference in nucleotide sequence variations per site between MGB probe and non-MGB probe regions (0.16 ± 0.14 vs. 0.074 ± 0.029, $P = 0.11$).

Discussion

In the present study, we used a novel HCV subgenotyping method based on real-time PCR [12] in Japanese patients infected with HCV genotype 1. Real-time PCR-based HCV subgenotyping method seems fair for differentiating the HCV subgenotypes 1a and 1b. But it might not be good enough for clinical practice, as HCV subgenotypes were only determined in 74 of 80 patients (92.5%), despite our study population being small. As direct-sequencing could only apply to cases in which the PCR products were obtained by RT-PCR methods, real-time PCR-based HCV subgenotyping method might be useful if the PCR products cannot be amplified.

Ultra-deep pyrosequencing is a promising technology for characterizing and detecting minor variants and drug resistant variants [15]. Pyrosequencing could be useful for cost-effective estimation of drugs for treatment and disease controls [16]. We

Table 4. Analysis of nucleotide sequence variations (%) in MGB probe segments by ultra-deep sequencing.

Refs.	Variants	Max	No. 4	No. 25	No. 37	No. 46	No. 47	No. 48	No.66	No. 74	No. 78
Con1	8912:A/G	100	***99.28***	0	***27.01***	***100***	0	0	0	NA	0.07
Con1	8913.5:-/G	30	0	0.05	0	0	***30***	0	0	NA	0
Con1	8915.5:T/C	0.52	0.52	0	0	0	0	0	0	NA	0
Con1	8915.5:-/A	70	0	0.83	0.26	0.29	***70***	0	0	NA	0
Con1	8917:A/G	99.84	0	0	0	***99.84***	***30***	0	0	NA	0.03
Con1	8918:A/G	99.92	0	0	0	0	0	***99.92***	0	NA	0
Con1	8919:A/G	0.52	0.52	0	0	0	0	0	0	NA	0
Con1	8920:A/G	2.33	***2.33***	0	0	0	0	0	0	NA	0
Con1	8921:A/G	85.79	***85.79***	0	0	0	0	0	0	NA	0
Con1	8921.5:-/G	0.44	0	0.4	0.07	0	***70***	0.08	0	NA	0.44
Con1	8922:G/A	1.11	***1.11***	0	0	0	0	0	0	NA	0
Con1	8922.5:-/G	1.09	0.97	0	0	0	0	0	0	NA	***1.09***
H77	8915:G/A	1.9	NA	NA	NA	NA	NA	NA	NA	***1.9***	NA

Reference sequences (Refs) were obtained from HCV subgenotype 1a (H77) and HCV subgenotype 1b (Con1). GenBank accession No.: H77, AF009606.1; Con1, AJ238799.1. Bold oblique indicates significant change (1%) from reference sequences. Number indicates the nucleotide changes that were identical in direct-sequencing by Sanger Method.

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also performed analysis of ultra-deep sequences in the probe region of HCV NS5B. In comparison with direct-sequencing by the Sanger method, we found other changes of nucleotide sequences in this region. It is possible that these variations might have some effects on the results by the real-time PCR-based HCV subgenotyping method. Our results support the previous studies showing that ultra-deep sequencing technologies are powerful tools for obtaining more profound insight into chronic HCV infection [17-19]. Our study also showed that analysis of ultra-deep sequences provided additional information that we could not obtain using direct-sequencing by the Sanger method. We also found minor sequence variations (1%), although we do not know whether these sequences are significant or what roles they play at this time. Further studies will be needed.

We estimated the prevalence of HCV subgenotype 1a at 1.2-2.5% of HCV genotype 1 patients in Japan. It is well known that some patients treated with telaprevir experienced viral breakthrough with telaprevir-resistant strains, most of which harbored the R155T/K mutations. Only a single nucleotide change is needed to produce the amino acid change at codon 155 in HCV subgenotype 1a, while two nucleotide changes are required in HCV subgenotype 1b. This might be one of the reasons why resistance mutation was selected more frequently in HCV subgenotype 1a than in HCV subgenotype 1b in the case of telaprevir use [20], although in the ELECTRON study of NS5B inhibitor sofosbuvir, no differential resistance was observed between genotypes 1a and 1b despite 89% of the subjects being the HCV genotype 1a population [21]. Of interest, it has been reported that there are different impacts of IL28B variants on the treatment response to SOC between HCV subgenotype 1a and 1b strains together with HIV co-infection [22]. It will be important to determine HCV subgenotype 1a or 1b before commencing treatment for HCV genotype 1.

It was reported that Versant HCV genotype assay (LIPA version.1 probe assay based on 5' UTR, Siemens, Tarrytown, NY, USA) could not determine HCV subgenotypes in 23% of HCV-infected patients in Brazil [12]. Thomas et al. reported that upgrading LIPA to the newer version 2.0 assay resulted in an increase in identification of genotype 1a by 18.5% [23]. Improved technology of HCV genotyping and subgenotyping could lead to accurate HCV genotype identification and HCV subtyping. Ultra-deep sequencing is useful for revealing the mutations in viral genome of HCV-infected individuals. However, it might not be useful as a method for differentiating HCV subgenotype 1a and 1b, although their combination as well as next-generation sequencing [24] could perhaps lead to better results in the determination of HCV subgenotypes. It could be used to find the resistant strain(s) of HCV before DAA treatment and mixed infection with different genotypes or subgenotypes of HCV, although Akuta et al. [25] reported that it was difficult to predict at baseline the emergence of telaprevir-resistant variants after commencement of therapy in prior non-responders of HCV genotype 1, even with the use of ultra-deep sequencing.

It was reported that phylogenetic analysis revealed different clusters between the HCV subgenotype 1b strains obtained in Japan and those in Brazil [26]. In fact, the distribution of HCV genotypes differs in various countries [27]. The discrepancy between the results of the present and previous studies [12] might be due to the differences between HCV subgenotype 1b strains in Japan and in Brazil.

In conclusion, the method for identifying HCV subgenotype 1a or 1b by the real-time PCR-based method in the HCV NS5B region was examined in Japanese patients infected with HCV genotype 1, and we estimated a 1.2-2.5% prevalence of HCV subgenotype 1a among HCV genotype 1 patients in Japan. We also analyzed the specific probe regions for HCV subgenotypes 1a and 1b, of which we could not determine HCV subgenotypes using specific probes, and uncovered the