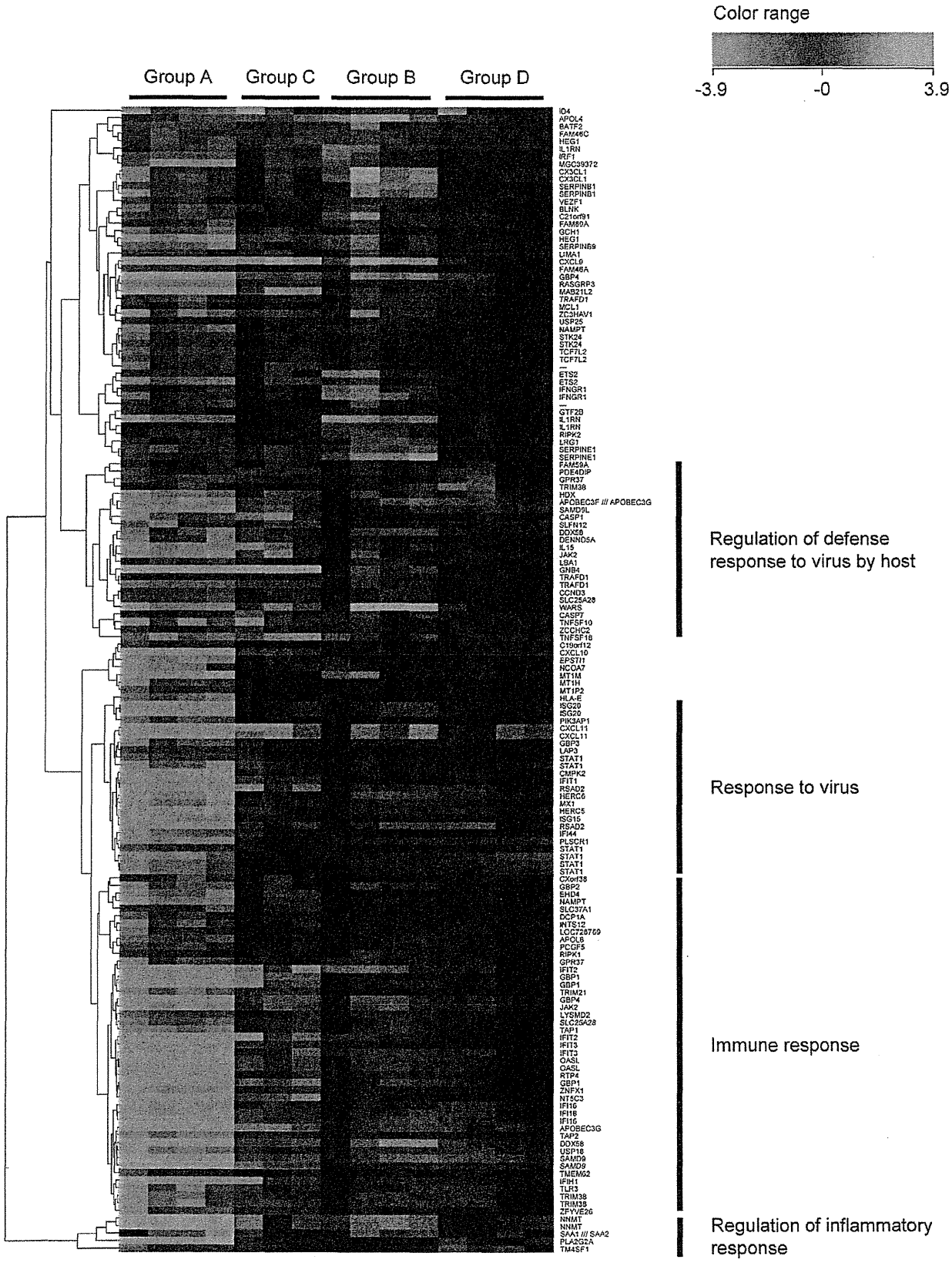
Color range
-3.9
-0
3.9
Group A
Group C
Group B
Group D
Regulation of defense response to virus by host
Response to virus
Immune response
Regulation of inflammatory response

Figure 5. Hierarchical clustering analysis of 152 genes associated with IFN administration with or without HCV infection. To analyze the effect of HCV infection on IFN response, gene expression ratios between Groups A and B (gene expression changes by IFN without HCV infection) and those between Groups C and D (gene expression changes by IFN with HCV infection) were compared in the 152 IFN-induced genes. 69.7% of the selected genes showed reduced IFN responsiveness following HCV infection.
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signaling were the most strongly up-regulated following HCV infection (Figure 3). These findings are mostly consistent with previous studies [36,37]. On the other hand, while no genes involved in lipid metabolism showed any significant induction by HCV infection in this study, Walters et al. reported that HCV-infected chimeric mice exhibited host-specific induction in the expression of lipid metabolism genes [35]. However, we used hepatocytes from a single donor, whereas Walters et al. used hepatocytes from multiple donors, so our results are not necessarily inconsistent with their findings that HCV infection causes induction of lipid metabolism genes in a host-specific manner.

Although several cDNA microarray analyses have also been performed using human liver tissues obtained after hepatic resection, the largest difference between human and chimeric mouse livers is the presence or absence of human lymphocytes. According to the previous report using human liver tissues, genes involved in the innate immune response, as well as cell cycle, growth and communication, were up-regulated by HCV infection [38]. In the present study using SCID-derived mice, genes involved in immune response (e.g. *OAS2*, *Mx1*, *IFI27* and *IFI44L*), cell cycle and growth (e.g. *HERC5*) and cell communication (e.g. *HLA-B*) were similarly up-regulated by HCV infection. However, *Apolipoprotein L*, *Cold autoinflammatory syndrome 1*, *CD97 antigen*, and *HLA-DQ*, which are mainly expressed in lymphocytes, were not observed to be up-regulated by HCV infection in the chimeric mice. These results demonstrate that the chimeric mouse model accurately reflects intracellular responses to HCV infection without the lymphocytic immune response.

Table 6. The top 20 genes in which IFN-induced up-regulation is inhibited following HCV infection.

Probe Set ID	Gene symbol	Fold change		P value
		HCV infection (-)	HCV infection (+)	
235175_at	GBP4	44.94	5.50	2.93E-07
231577_s_at	GBP1	24.60	4.89	6.15E-07
218943_s_at	DDX58	32.19	5.56	1.26E-05
226702_at	CMPK2	12.50	2.13	2.35E-05
225973_at	TAP2	7.07	2.84	3.31E-05
229450_at	IFIT3	20.66	2.74	6.37E-05
217739_s_at	NAMPT	5.91	1.95	6.51E-05
213797_at	RSAD2	69.70	4.50	7.01E-05
210797_s_at	OASL	31.59	3.53	1.02E-04
218508_at	DCP1A	3.56	1.65	1.36E-04
228531_at	SAMD9	18.65	5.87	1.45E-04
204804_at	TRIM21	5.37	2.69	1.47E-04
219209_at	IFIH1	10.05	4.69	1.67E-04
219684_at	RTP4	16.38	2.55	1.98E-04
239186_at	MGC39372	7.61	2.57	2.27E-04
219211_at	USP18	8.72	3.74	2.40E-04
225076_s_at	ZNFX1	10.40	2.91	2.91E-04
204698_at	ISG20	31.29	3.33	3.00E-04
223192_at	SLC25A28	5.05	2.20	3.25E-04
228439_at	BATF2	4.59	1.83	3.28E-04

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To verify the microarray results, expression data were compared with previously published microarray data on the GEO website (http://www.ncbi.nlm.nih.gov/geo/). Previously published microarray data showed up-regulation of *IGFBP7*, *IFI27*, *HLA-B*, and *CD74* in HCV-infected liver tissues compared to non-infected liver tissues (fold changes were 2.1, 2.2, 2.1 and 2.3, respectively) [39]. Likewise, we found that *IFI27*, *HLA-B*, and *CD74* were up-regulated following HCV infection (fold changes were 3.6, 3.3, and 6.6, respectively). These three genes are associated with MHC class I activity, suggesting that intra-cellular immunity in human hepatocytes was activated following HCV infection both in human subjects and in chimeric mouse livers. Metallothionein 1G (*MT1G*) expression was also found to be up-regulated by HCV infection in both the current and published studies [39,40]. Although metallothionein isoforms are associated with collagen deposition [41], members of the metallothionein family may be up-regulated and induce liver fibrosis in response to HCV infection.

In this study, genes associated with Organismal Injury and Abnormalities were found to be up-regulated in response HCV infection (Table 3), and some genes in this category, such as *CXCL9*, *CXCL10* and *IFIT3*, maintained high IFN responsiveness under HCV infection (Table 8). These results suggest that protective responses to fibrosis or hepatic injury were activated at the start of HCV infection and remained activated until complete eradication of HCV from hepatocytes was achieved.

Secondly, we compared gene expression profiles between groups A (without IFN treatment) and B (with IFN treatment) to evaluate IFN response without HCV infection. IFN-α stimulates the intracellular IFN-signaling cascade after binding to the IFN-α receptor and mediates the transcriptional activation of IFN-stimulated genes [42–47]. More than 3.0-fold up-regulation was observed 6hrs after IFN treatment in 152 genes. Known ISGs such as those in the *CXCL* family (*CXCL9*, *CXCL10* and *CXCL11*), the *IFIT* family (*IFIT2* and *IFIT3*) and the *APOBEC* family (*APOBEC3G*) were included among the top 20 genes up-regulated following IFN treatment (Table 4). The *APOBEC* family is well known to have anti-viral effects by inducing genomic hypermutation in human immunodeficiency virus and hepatitis B virus [48–57]. *APOBEC3G* expression has been reported to be elevated in patients infected with HCV [58], although it is not clear whether *APOBEC3G* can block HCV replication. On the other hand, *CXCL9* and *IFIT3* were reported to relate to liver fibrosis in chronic hepatitis C patients. Serum *CXCL9* concentrations correlated with the levels of fibrosis in chronic hepatitis C patients, and *CXCL9* has been shown to exert anti-fibrotic effects *in vitro* and *in vivo* [59]. *IFIT3* expression is also reportedly up-regulated in the transition from mild to moderate fibrosis [60]. The results of this study suggest that IFN treatment might lead not only to HCV eradication but also help to prevent and repair liver fibrosis by inducing these key molecules.

We focused on the 152 genes up-regulated (> 3.0 fold) as a result of IFN administration and evaluated the effect of HCV infection on IFN response among these genes. As shown in Table 8, although several ISGs still showed high response to IFN

Table 7. The top 5 canonical pathways associated with the 106 genes in which IFN response is suppressed by HCV infection.

Category	P value	Ratio	Associated genes
Interferon Signaling	4.41E-11	7/30 genes	IFIT1, IFIT3, IFNGR1, IRF1, MX1, STAT1, TAP1
Type I Diabetes Mellitus Signaling	1.08E-04	5/119 genes	HLA-E, IFNGR1, IRF1, RIPK1, STAT1
Antigen Presentation Pathway	5.29E-04	3/39 genes	HLA-E, TAP1, TAP2
Primary Immunodeficiency Signaling	1.64E-03	3/63 genes	BLNK, TAP1, TAP2
Activation of IRF by Cytosolic Pattern Recognition Receptors	2.95E-03	3/73 genes	ISG15, RIPK1, STAT1

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treatment in the presence of HCV infection, 7 genes in the IFN Signaling pathway became unresponsive (Table 6). Reduction in IFN responsiveness was also observed for *STAT1* (4.27 fold in the absence of HCV to 2.29 fold in the presence of HCV, $P = 4.04 \times 10^{-4}$), as well as 5 of 7 genes downstream of *STAT1* (*IFIT1*, *IFIT3*, *IRF1*, *MX1*, and *TAP1*). As shown in Figure 3, IFN

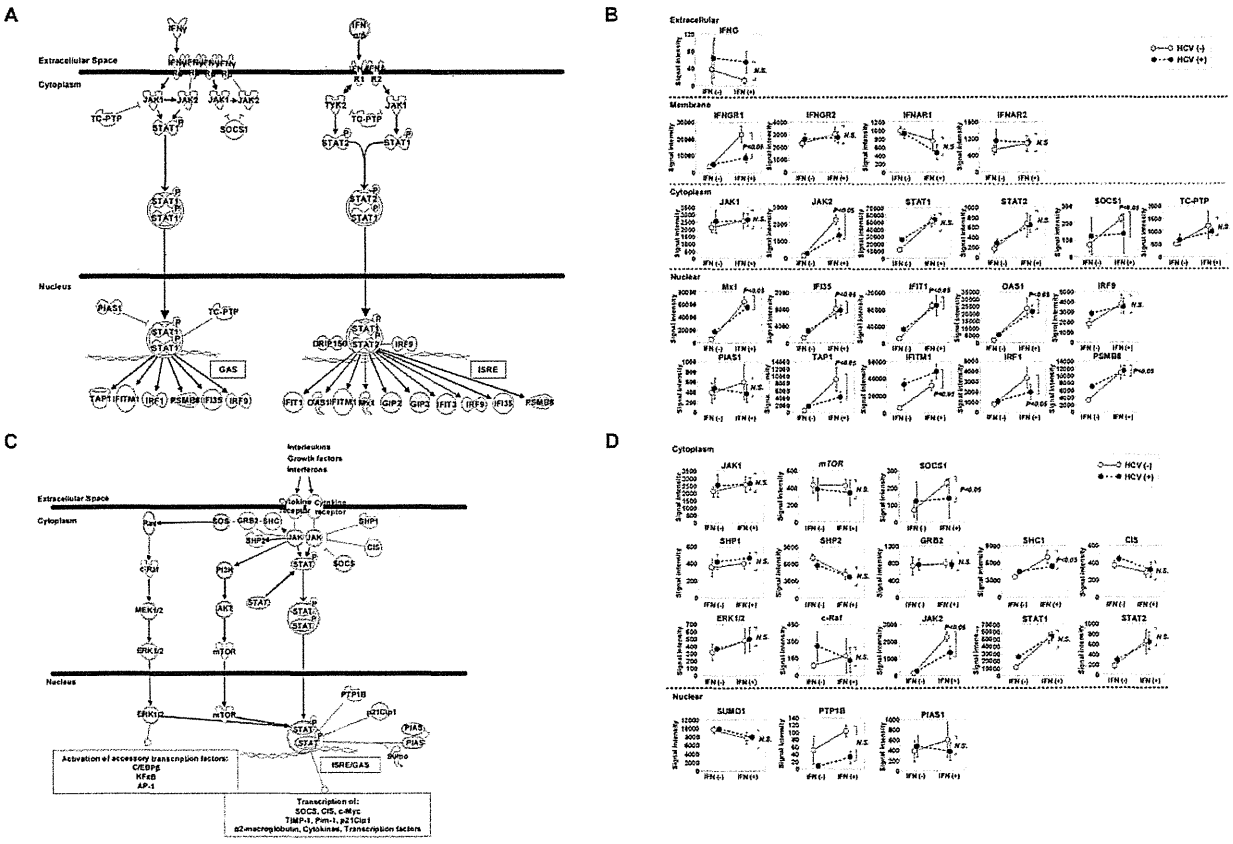

Figure 6. Changes in expression of genes in the IFN and JAK-STAT signaling pathways under HCV infection and/or IFN administration. A) An overview of the IFN signaling pathway consisting of 26 representative genes is shown. Genes illustrated as gray shapes were not included in this study. B) Relative expression levels of genes with/without HCV infection and/or IFN administration were plotted (closed dots: with HCV infection; open dots: without HCV infection) using microarray data. The slopes of the dashed and solid lines represent IFN responsiveness with and without HCV infection, respectively. In 21 of the 22 examined genes in the IFN signaling pathway, signal intensities increased and IFN responsiveness was repressed following HCV infection. Student's *t*-test was used for statistical analysis. C) An overview of the JAK-STAT signaling pathway consisting of 22 representative gene products is shown. Genes illustrated as gray shapes were not included in this study. D) Relative expression levels of genes with/without HCV infection and/or IFN administration were plotted using microarray data (closed dots: with HCV infection; open dots: without HCV infection). Signal intensities increased following HCV infection in 16 of 22 genes in the JAK-STAT signaling pathway, and IFN response was suppressed in 9 genes. Statistical analysis was performed using Student's *t*-test.
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Table 8. The 33 genes that remained more than 3-fold up-regulated following IFN treatment in HCV-infected mice.

ID	Symbol	Location	Type(s)
214995_s_at	APOBEC3F	unknown	enzyme
204205_at	APOBEC3G	Nucleus	enzyme
206011_at	CASP1	Cytoplasm	peptidase
204533_at	CXCL10	Extracellular Space	cytokine
210163_at	CXCL11	Extracellular Space	cytokine
203915_at	CXCL9	Extracellular Space	cytokine
218943_s_at	DDX58	Cytoplasm	enzyme
231577_s_at	GBP1 (includes EG:2633)	Cytoplasm	enzyme
235175_at	GBP4 (includes EG:115361)	Cytoplasm	enzyme
225710_at	GNB4	Plasma Membrane	enzyme
1553646_at	HDX	unknown	other
213069_at	HEG1	unknown	other
206332_s_at	IFI16	Nucleus	transcription regulator
219209_at	IFIH1	Nucleus	enzyme
217502_at	IFIT2	unknown	other
204747_at	IFIT3	Cytoplasm	other
205992_s_at	IL15	Extracellular Space	cytokine
204698_at	ISG20	Nucleus	enzyme
205841_at	JAK2	Cytoplasm	kinase
210302_s_at	MAB21L2	unknown	other
223298_s_at	NT5C3	Cytoplasm	phosphatase
205660_at	OASL	unknown	enzyme
205801_s_at	RASGRP3	Cytoplasm	other
242625_at	RSAD2	unknown	enzyme
228531_at	SAMD9	unknown	other
230036_at	SAMD9L	unknown	other
219885_at	SLFN12	Nucleus	enzyme
206271_at	TLR3	Plasma Membrane	transmembrane receptor
214329_x_at	TNFSF10	Extracellular Space	cytokine
221371_at	TNFSF18	Extracellular Space	cytokine
203610_s_at	TRIM38	unknown	other
219211_at	USP18	Cytoplasm	peptidase
200629_at	WARS	Cytoplasm	enzyme

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signaling was activated in the presence of HCV, and the expression of *STAT1* was more than 3.0 fold up-regulated by HCV infection (data not shown). *STAT1* expression was highest in mice with both HCV infection and IFN treatment, but downstream genes such as *MX1*, *IFIT1* and *IFIT3* showed reduced IFN response. Sarasin-Filipowicz et al. reported that IFN-induced *STAT1* phosphorylation was stronger in rapid responders than in non-rapid responders [61]. Reduced induction of genes downstream of *STAT1* by IFN under HCV infection might reflect reduced phosphorylation of *STAT1*, although we did not quantify *STAT1* phosphorylation in this study.

Recently, an *IL-28B* genetic polymorphism strongly associated with response to IFN-α plus ribavirin combination therapy [12], as well as with hepatic ISG expression [62], was identified. Further studies using chimeric mice transplanted with hepatocytes carrying different genotypes of candidate genes such as *IL-28B* will be important in order to elucidate possible mechanisms underlying host-specific responses.

In conclusion, we performed cDNA microarray analysis using HCV-infected human hepatocyte chimeric mice, which allowed us to analyze the direct effects of IFN treatment and HCV infection without the confounding effects of the lymphocytic immunological response. These results might provide molecular insights into possible mechanisms used by HCV to evade IFN-induced immune responses, as well as suggest novel therapeutic targets and a potential new indication for interferon therapy. Further analysis of the genes identified in our study would be worthwhile in order to improve efficacy of the therapy for chronic hepatitis C.

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Author Contributions

Conceived and designed the experiments: MT YF NH MI ST KC. Performed the experiments: MT YF NH FM TT. Analyzed the data: MT YF YZ MO TK HA DM. Contributed reagents/materials/analysis tools: MT NH MI FM TT KC. Wrote the paper: MT YF ST HO CNH KC.

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BRIEF REPORT

Effects of Hepatitis B Virus Infection on the Interferon Response in Immunodeficient Human Hepatocyte Chimeric Mice

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Complementary DNA microarray analysis of human livers cannot exclude the influence of the immunological response. In this study, complementary DNA microarray analysis was performed under immunodeficient conditions with human hepatocyte chimeric mice, and gene expression profiles were analyzed by hepatitis B virus (HBV) infection and/or interferon treatment. The expression levels of 183 of 525 genes upregulated by interferon treatment were significantly suppressed in response to HBV infection. Suppressed genes were statistically significantly associated with the interferon signaling pathway and pattern recognition receptors in the bacteria/virus recognition pathway ($P = 1.0 \times 10^{-8}$ and $P = 1.2 \times 10^{-8}$, respectively). HBV infection attenuated virus recognition and interferon response in hepatocytes, which facilitated HBV escape from innate immunity.

Chronic hepatitis B virus (HBV) infection is associated with the development of virus-related liver diseases, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Interferon α (IFN-α) has been used for the treatment of chronic hepatitis B, and many large clinical trials and meta-analyses have demonstrated the effectiveness of interferon [1–3]. However, the effect of IFN-α therapy is unsatisfactory, and the molecular basis for tolerance to IFN-α is not clearly defined.

DNA microarray technology has enabled genome-wide analysis of gene transcript levels with the use of clinical tissues and animal models, which has yielded insights into the molecular features of several liver diseases [4–6]. However, it has been difficult to determine whether the changes in gene expression were caused by viral interference or by the human immune response, because all of these studies that used clinical and experimental samples were analyzed under the influence of adaptive immune responses. Recently, Mercer and colleagues developed a human hepatocyte chimeric mouse model [7]. These mice were derived from severe combined immunodeficiency (SCID) mice, which are severely immunocompromised, and the mouse liver cells were extensively replaced with human hepatocytes [7, 8]. With the use of this chimeric mouse model, in which HBV can continuously infect human hepatocytes, the effect of drugs and the response of viral infection can be analyzed in human hepatocytes under immunodeficient conditions [9]. In this study, we performed microarray analysis with human hepatocyte chimeric mouse livers to assess the direct impacts of HBV infection and IFN treatments on gene expression profiles. We successfully demonstrated that HBV infection attenuated the expression of IFN-stimulating genes under immunodeficient conditions, which suggests that HBV proteins might afford escape mechanisms from cellular innate immunity.

METHODS

A serum sample was obtained from a HBV carrier after obtaining written informed consent for the donation and evaluation of the blood sample. The inoculum was positive for Hepatitis B surface and Hepatitis B e antigens, with slightly elevated levels of serum alanine aminotransferase and high-level viremia (HBV DNA load, 7.1 log copies/mL). The studied patient was infected with HBV genotype C. The experimental protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the Hiroshima University Hospital ethical committee (approval ID: D08-9).

The uPA$^{+/+}$/SCID$^{+/+}$ mice were prepared and the human hepatocytes were transplanted as described elsewhere [8]. The experiments were performed in accordance with the guidelines of the local committee for animal experiments at Hiroshima University.

Sixteen chimeric mice, in which >90% of the liver tissue was replaced with human hepatocytes, were divided into

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4 experimental groups. Group A contained 4 mice that were neither infected with HBV nor treated with IFN. Group B consisted of 3 mice that were treated with IFN-α for 6 h (7,000 IU per gram of body weight) just before being humanely killed but were not infected with HBV. Mice in groups C and D were inoculated via the mouse tail vein with human serum containing 6×10^6 copies of HBV. After inoculation, we collected mouse serum samples every 2 weeks and analyzed HBV DNA titers by real-time polymerase chain reaction (PCR) and human albumin levels by means of a human albumin enzyme-linked immunosorbent assay quantitation kit (Bethyl Laboratories), as described elsewhere [9]. Virus and human albumin titer levels are shown in Supplementary data 1. All 9 mice developed measurable viremia 4 weeks after inoculation. Eight weeks after inoculation, 4 of the 9 infected mice (group C) were humanely killed without IFN treatment and the remaining 5 mice (group D) were humanely killed after 6 h of IFN-α treatment (7,000 IU per gram of body weight). The mice were infected, had serum samples extracted, and were killed humanely under ether anesthesia, as described elsewhere [8].

All 16 chimeric mice were killed humanely, and human hepatocytes were finely dissected from the mouse livers and stored in liquid nitrogen after submerging in RNA later solution (Applied Biosystems). Total RNA was extracted with TRIzol reagent (Invitrogen) and labeled with cyanine 3 by use of a low RNA input linear amplification kit (Agilent Technologies) after amplification. Cyanine-3–labeled complimentary RNA was hybridized to a 44-K whole human genome oligo microarray (Agilent). Detailed protocols are described in Supplementary data 2.

Gene expression profiles were analyzed using GeneSpring GX software (version 10.0.2; Tomy Digital Biology). The detailed protocol is described in Supplementary data table 3. Complete linkage hierarchical clustering analysis was applied using Euclidean distance, and differentially expressed genes were annotated using information from the Gene Ontology (GO) Consortium. Global molecular networks and comparisons of canonical pathways were generated using Ingenuity Pathway Analysis (IPA) software (version 8.6; Ingenuity Systems).

Total RNA was extracted from the implanted human hepatocytes in the mouse livers by use of an RNeasy mini kit (Qiagen) and was reverse transcribed. The selected messenger RNA (mRNA) was quantified by real-time PCR using the 7300 real-time PCR system (Applied Biosystems), and the expression of glyceraldehyde-3-phosphate dehydrogenase served as a control. The amplification protocol and primer sequences are described in Supplementary data 4 and 5.

RESULTS

To analyze the direct effects of IFN in human hepatocytes, we compared the gene expression profiles between groups A (mice without IFN treatment) and B (mice with IFN treatment). Of the 1403 genes that remained after screening with the Welch *T* test, 685 genes showed a >3.0-fold change between groups. Of these 685 genes, 525 genes were up-regulated and the other 160 genes down-regulated by IFN. The top 20 IFN-regulated genes are listed in Supplementary data table 6. GO analysis revealed that 8 (40%) of the top 20 genes that were upregulated with IFN treatment were related to immune response.

To analyze the effect of HBV infection in human hepatocytes, we compared the gene expression profiles between groups A (mice without HBV infection) and C (mice with HBV infection). Among the 1,714 genes hat remained after screening, 373 genes showed a >3.0-fold change between groups. Of these 373 genes, 159 genes were up-regulated and the other 214 genes down-regulated by HBV. The top 20 HBV-regulated genes are listed in Supplementary data table 7. Several oncogenic genes such as growth differentiation factor 15 and glial cell derived neurotrophic factor were included in the top group. Most of the top 20 genes that were downregulated with HBV infection were associated with transcriptional regulation.

To examine whether HBV infection may alter the effect of IFN response in human hepatocytes, we compared gene expression profiles among all groups. As mentioned above, 525 genes were upregulated by >3.0-fold by IFN in the absence of HBV infection. A comparison of groups C (mice with HBV infection but no IFN treatment) and D (mice with both HBV infection and IFN treatment) revealed that 183 (34.9%) of the 525 genes showed statistically significantly reduced IFN response with HBV infection ($P < .01$) (Supplementary data 8A). The top 20 genes in which IFN response was significantly changed by HBV infection are shown in Table 1. The mRNA expression levels of 11 selected genes among the 183 genes with reduced IFN response were also analyzed by real-time PCR, and the reductions in IFN response by HBV infection were verified (Supplementary data 8B). Additionally, we used IPA software to analyze the influence of HBV infection on the IFN response of these 183 genes by means of a pathway-oriented approach. Pathway analysis revealed that several pathways were affected by HBV infection (Table 2). The IFN response was statistically significantly attenuated by HBV infection in the pathways related to IFN signaling and pattern recognition of bacteria and viruses ($P = 1.0 \times 10^{-8}$ and $P = 1.2 \times 10^{-8}$, respectively).

DISCUSSION

Elsewhere we have demonstrated a human hepatocyte chimeric mouse model that can be chronically infected with hepatitis B and C viruses [9–11]. This mouse model facilitates analysis of the effect of viral infection and the response to medication under immunodeficient conditions. In this study, we performed complementary DNA microarray analysis using the chimeric mouse model and obtained gene expression profiles to analyze

Table 1. Genes With Interferon Responsiveness Downregulated by Hepatitis B Virus (HBV) Infection

Gene symbol	GenBank accession no.	Function	Fold change in expression level		*P*
			Without HBV infection	With HBV infection	
ENST00000322831	None	Unknown	4.52	−1.45	4.15×10^{-7}
AA593970	AA593970	EST	9.70	1.61	5.58×10^{-7}
THC2533996	None	Unknown	3.74	−2.50	6.97×10^{-7}
LOC388532	None	Unknown	3.11	−2.48	1.61×10^{-6}
ZNF267	NM_003414	Transcription regulator	7.66	1.79	2.30×10^{-6}
ZNF217	NM_006526	Transcription regulator	3.69	1.03	3.62×10^{-6}
CRSP3	NM_015979	Transcription regulator	7.50	−1.02	4.06×10^{-6}
MGC39372	BC025340	Hypothetical protein	30.92	7.03	5.74×10^{-6}
BF972140	BF972140	EST	16.91	4.71	5.78×10^{-6}
LOC731599	XR_015536	Hypothetical protein	3.17	−4.18	8.58×10^{-6}
LOC645676	AK126559	Hypothetical protein	3.76	1.35	9.13×10^{-6}
THC2650457	None	Unknown	78.07	6.28	1.29×10^{-5}
ZNF24	NM_006965	Transcription regulator	3.69	1.36	1.64×10^{-5}
CCDC68	NM_025214	Unknown	5.88	−2.83	1.89×10^{-5}
SP110	NM_004510	Transcription regulator	5.00	10.77	2.00×10^{-5}
FLJ21272	AK024925	Hypothetical protein	14.70	2.49	3.18×10^{-5}
PLEKHF1	NM_024310	Unknown	6.65	1.84	4.70×10^{-5}
AK026418	AK026418	Unknown	9.50	2.58	5.02×10^{-5}
hCG_1790262	XM_001133847	Unknown	3.13	−2.94	6.25×10^{-5}
CEBPD	NM_005195	Transcription regulator	8.16	1.56	7.03×10^{-5}
FLJ20273	NM_019027	RNA binding	3.37	1.11	7.11×10^{-5}

NOTE. *P* values were analyzed by the Welch *T* test. *CEBPD,* CCAAT/enhancer binding protein (C/EBP) delta; *CCDC68,* coiled-coil domain containing 68; *CRSP3,* mediator complex subunit 23 (*MED23*); EST, expressed sequence tag; *FLJ20273,* RNA binding motif protein 47 (*RBM47*); *PLEKHF1,* pleckstrin homology domain containing, family F (with FYVE domain) member 1; *SP110,* SP110 nuclear body protein; *ZNF24,* zinc finger protein 24; *ZNF217,* zinc finger protein 217; *ZNF267,* zinc finger protein 267.

the direct influence of HBV infection and IFN-α treatment on human hepatocytes.

To avoid contamination with mouse tissue, human hepatocyte chimeric mice, in which liver tissue is largely (>90%) replaced by human hepatocytes, were used in the present study. However, a small amount of mouse-derived cells, such as interstitial cells, bile duct cells, and vascular cells, still remain in the chimeric mouse livers. Because of high homology between the human and mouse genomes, the signals from microarray analyses may be influenced by cross-hybridization with mouse mRNA. It is difficult to produce $uPA^{+/+}/SCID^{+/+}$ mice >10 weeks old without hepatocyte transplantation, and a previous study demonstrated that it is feasible to use microarray analysis in a functional genomics analysis of chimeric mice [12]. Therefore, to compensate for the contamination, the mice in group A, which were neither infected with HBV nor treated with IFN, were used as negative controls.

To analyze the effect of IFN treatment, we compared gene expression profiles between groups A (mice without IFN treatment) and B (mice with IFN treatment); 525 genes with >3.0-fold upregulation following IFN treatment were observed. Among them, chemokine (C-X-C motif) ligand 9, chemokine (C-X-C motif) ligand 10, and chemokine (C-X-C motif) ligand 11, which promote T cell adhesion, were remarkably highly induced with IFN treatment (Supplementary data table 6) [13]. These results suggest that the antiviral effects of IFN might involve not only direct activation of IFN-stimulated proteins such as myxovirus resistance protein A and double strand RNA-dependent protein kinase but also activation of immunity via chemokines.

Second, we compared the profiles between groups A (mice without HBV infection) and C (mice with HBV infection). As shown in Supplementary data table 7, more than half (12) of the top 20 genes upregulated by HBV infection localized to the cell membrane or the extracellular region, but 14 (70%) of the 20 downregulated genes localized to the nucleus. In addition, GO analysis demonstrated that genes related to cell cycle and DNA modification were affected by HBV infection. We speculate that HBV infection promotes cell growth and DNA damage in the hepatocyte nucleus and activates the immune response in the cytoplasm. From the clinical standpoint, some healthy HBV carriers develop hepatocellular carcinoma without chronic hepatitis or cirrhosis. The present results strongly support this observation, showing that most of the affected genes are known to be associated with carcinogenesis.

Clinically, HBV is known to develop tolerance to IFN treatment in patients with chronic hepatitis B, although the mechanism is not clear. We analyzed the IFN response with and

Table 2. Pathway Analysis of 183 Interferon-Induced Genes With Interferon Responsiveness Downregulated by Hepatitis B Virus Infection

Canonical pathways	*P*	Genes
Interferon signaling	1.00×10^{-8}	*IFIT3, SOCS1, IFIT1, MX1, IFNGR1, JAK2, STAT1, TAP1, IRF1*
Role of pattern recognition receptors in recognition of bacteria and viruses	1.20×10^{-8}	*IL12A, OAS2, OAS3*(includes EG:4940), *IFIH1, PIK3R3, TLR4, NOD2, TICAM1, DDX58, CASP1, NOD1, TLR3, RIPK2*
Type 1 diabetes mellitus signaling	2.00×10^{-4}	*SOCS1, IL12A, RIPK1, GAD1, SOCS6, SOCS2, IFNGR1, JAK2, STAT1, IRF1*
Prolactin signaling	2.70×10^{-4}	*PIK3R3, SOCS1, SOCS6, SOCS2, NMI, JAK2, STAT1, IRF1*
TREM1 signaling	3.50×10^{-4}	*TLR4, NOD2, ICAM1, CASP1, JAK2, TLR3, CASP5*
Production of nitric oxide and reactive oxygen species in macrophages	3.90×10^{-4}	*PIK3R3, TLR4, RND3, PPP2R2A, PPM1J, RHOU, IFNGR1, MAP3K8, IRF8, JAK2, STAT1, IRF1*
Pathogenesis of multiple sclerosis	1.10×10^{-3}	*CXCL10, CXCL9, CXCL11*
Activation of IRF by cytosolic pattern recognition receptors	2.60×10^{-3}	*IFIH1, RIPK1, DDX58, STAT1, IFIT2, ISG15*
Dendritic cell maturation	2.60×10^{-3}	*B2M, PIK3R3, TLR4, ICAM1, IL12A, IL1RN, IRF8, JAK2, TLR3, STAT1*
Interleukin 12 signaling and production in macrophages	3.60×10^{-3}	*PIK3R3, TLR4, IL12A, IFNGR1, MAP3K8, IRF8, STAT1, IRF1*
Sphingosine-1-phosphate signaling	3.60×10^{-3}	*PIK3R3, S1PR2, RND3, CASP1, RHOU, CASP4, CASP7, CASP5*
JAK-STAT signaling	4.00×10^{-3}	*PIK3R3, SOCS1, SOCS6, SOCS2, JAK2, STAT1*
Growth hormone signaling	4.70×10^{-3}	*PIK3R3, SOCS1, SOCS6, SOCS2, JAK2, STAT1*
Retinoic acid mediated apoptosis signaling	8.50×10^{-3}	*TNFRSF10B, PARP8, TNFSF10, TIPARP, IRF1*

NOTE. *B2M,* beta-2-microglobulin; *CASP1,* caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase); *CASP4,* caspase 4, apoptosis-related cysteine peptidase; *CASP5,* caspase 5, apoptosis-related cysteine peptidase; *CASP7,* caspase 7, apoptosis-related cysteine peptidase; *CXCL9,* chemokine (C-X-C motif) ligand 9; *CXCL10,* chemokine (C-X-C motif) ligand 10; *CXCL11,* chemokine (C-X-C motif) ligand 11; *DDX58,* DEAD (Asp-Glu-Ala-Asp) box polypeptide 58; *GAD1,* glutamate decarboxylase 1 (brain, 67kDa); *ICAM1,* intercellular adhesion molecule 1; *IFIH1,* interferon induced with helicase C domain 1; *IFIT1,* interferon-induced protein with tetratricopeptide repeats 1; *IFIT2,* interferon-induced protein with tetratricopeptide repeats 2; *IFIT3,* interferon-induced protein with tetratricopeptide repeats 3; *IFNGR1,* interferon gamma receptor 1; *IL1RN,* interleukin 1 receptor antagonist; *IL12A,* interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35); IRF, interferon regulatory factor; *IRF1,* interferon regulatory factor 1; *IRF8,* interferon regulatory factor 8; *ISG15,* ISG15 ubiquitin-like modifier; *JAK2,* Janus kinase 2; *MAP3K8,* mitogen-activated protein kinase kinase kinase 8; *MX1,* myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse); *NMI,* N-myc (and STAT) interactor; *NOD1,* nucleotide-binding oligomerization domain containing 1; *NOD2,* nucleotide-binding oligomerization domain containing 2; *OAS2,* 2′-5′-oligoadenylate synthetase 2, 69/71kDa; *OAS3,* 2′-5′-oligoadenylate synthetase 3, 100kDa; *PARP8,* poly (ADP-ribose) polymerase family, member 8; *PIK3R3,* phosphoinositide-3-kinase, regulatory subunit 3 (gamma); *PPM1J,* protein phosphatase, Mg2+/Mn2+ dependent, 1J; *PPP2R2A,* protein phosphatase 2, regulatory subunit B, alpha; *RHOU,* ras homolog gene family, member U; *RIPK1,* receptor (TNFRSF)-interacting serine-threonine kinase 1; *RIPK2,* receptor-interacting serine-threonine kinase 2; *RND3,* Rho family GTPase 3; *S1PR2,* sphingosine-1-phosphate receptor 2; *SOCS1,* suppressor of cytokine signaling 1; *SOCS2,* suppressor of cytokine signaling 2; *SOCS6,* suppressor of cytokine signaling 6; *STAT1,* signal transducer and activator of transcription 1, 91kDa; *TAP1,* transporter 1, ATP-binding cassette, sub-family B (MDR/TAP); *TICAM1,* Toll-like receptor adaptor molecule 1; *TIPARP,* TCDD-inducible poly(ADP-ribose) polymerase; *TLR3,* Toll-like receptor 3; *TLR4,* Toll-like receptor 4; *TNFRSF10B,* tumor necrosis factor receptor superfamily, member 10b; *TNFSF10,* tumor necrosis factor (ligand) superfamily, member 10; *TREM1,* triggering receptor expressed on myeloid cells 1.

without HBV infection, focusing on the 525 upregulated genes with IFN treatment and using all obtained gene expression profiles. Interestingly, 61.3% of the extracted genes maintained an IFN response, but in 34.9% of those genes, IFN responses were attenuated by HBV infection (Supplementary data 8A). Genes corresponding to interferon signaling, including suppressor of cytokine signaling 1 (*SOCS1*) and interferon regulatory factor 1, and those corresponding to pattern recognition of bacteria and viruses, including nucleotide-binding oligomerization domain containing 1 (*NOD1*) and receptor-interacting serine-threonine kinase 2 (*RIPK2*), were statistically significantly associated with HBV-mediated attenuation to IFN response ($P = 1.0 \times 10^{-8}$ and $P = 1.2 \times 10^{-8}$, respectively). According to these results, HBV infection significantly up-regulated *SOCS1* expression and reduced the IFN responsiveness of *SOCS1*. Thus, *SOCS1* might support chronic infection of HBV in escaping the effects of innate immunity or IFN therapy. On the other hand, genes involved in recognition of viral infection were also inhibited following HBV infection. Both *NOD1* and *RIPK2* are related to innate and adaptive immune responses [14, 15]. We speculated that inhibition of *NOD1* or *RIPK2* expression facilitates HBV survival. Although further study is needed, these results may have important implications for the mechanisms of viral escape from innate immunity.

In conclusion, we performed complementary DNA microarray analysis using human hepatocyte chimeric mice. With this system, we could analyze the direct effects of IFN treatment and HBV infection without the confounding effects of the lymphocyte immunological response and obtained evidence that HBV infection attenuated the virus recognition and IFN response in

hepatocytes, by which means HBV could evade innate immune detection and response.

Supplementary Data

Supplementary data are available at *The Journal of Infectious Diseases* online.

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Research Article

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Elimination of hepatitis C virus by short term NS3-4A and NS5B inhibitor combination therapy in human hepatocyte chimeric mice

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Background & Aims: The current treatment regimen for chronic hepatitis C virus (HCV) infection is peg-interferon plus ribavirin combination therapy. The majority of developing therapeutic strategies also contain peg-interferon with or without ribavirin. However, interferon is expensive and sometimes intolerable for some patients because of severe side effects.

Methods: Using human hepatocyte chimeric mice, we examined whether a short term combination therapy with the HCV NS3-4A protease inhibitor telaprevir and the RNA polymerase inhibitor MK-0608 with or without interferon eradicates the HCV from infected mice. The effect of telaprevir and MK-0608 combination therapy was examined using subgenomic HCV replicon cells.

Results: Combination therapy with the two drugs enhanced inhibition of HCV replication compared with either drug alone. In *in vivo* experiments, early emergence of drug resistance was seen in mice treated with either telaprevir or MK-0608 alone. However, emergence was prevented by the combination of these drugs. Mice treated with a triple combination therapy of telaprevir, MK-0608, and interferon became negative for HCV RNA soon after commencement of the therapy, and HCV RNA was not detected in serum of these mice 12 weeks after cessation of the therapy. Furthermore, all mice treated with a high dose telaprevir and MK-0608 combination therapy for 4 weeks became negative for HCV RNA 1 week after the beginning of the therapy and remained negative after 18 weeks.

Conclusions: Eradication of HCV from mice with only 4 weeks of therapy without interferon points the way to future combination therapies for chronic hepatitis C patients.

Keywords: NS3-4A protease inhibitor; NS5B RNA polymerase inhibitor; Human hepatocyte chimeric mouse; Interferon.

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Abbreviations: HCV, hepatitis C virus; IFN, interferon; RBV, ribavirin; SVR, sustained virological response; STAT-C, specifically targeted antiviral therapy for HCV; uPA, urokinase-type plasminogen activator; SCID, severe combined immunodeficiency; RT-PCR, reverse transcript-polymerase chain reaction; HSA, human serum albumin.

Introduction

Chronic hepatitis C virus (HCV) infection is a leading cause of cirrhosis, liver failure, and hepatocellular carcinoma [1,2]. The current standard treatment for patients chronically infected with HCV is the combination of peg-interferon (PEG-IFN) and ribavirin (RBV) [3–5]. However, this treatment results in a sustained viral response (SVR), defined as negative for HCV RNA 24 weeks after cessation of the therapy, in only about 50% of patients with genotype 1 HCV infection with high viral load [3–5]. In view of the lack of effectiveness of the current therapy, many molecules have been tested for development of novel anti-HCV therapies. Recently, a number of new selective inhibitors of HCV proteins, the so-called STAT-C (specifically targeted antiviral therapy for HCV) inhibitors, have been in development. The HCV NS3-4A protease inhibitor and the NS5B polymerase inhibitor, as well as an inhibitor of NS5A function, have been demonstrated to have potent anti-HCV effects and have proceeded to clinical trials [6].

Although the anti-viral effect of these drugs is quite potent, monotherapy using these drugs results in early emergence of drug-resistant strains [7,8]. Accordingly, these drugs are used in combination with PEG-IFN and RBV. However, because IFN-treatment is expensive and is frequently associated with serious adverse events, such as cytopenias, rash/itching, alopecia, and

mental disorders [3–5,9], a new treatment strategy, especially one that does not use IFN, is needed for chronic hepatitis C patients.

The immunodeficient urokinase-type plasminogen activator (uPA) mouse permits repopulation of the liver with human hepatocytes that can be infected with HCV [10]. We and other groups reported that the human hepatocyte chimeric mouse is useful for evaluating anti-HCV drugs such as IFN-alpha and the NS3-4A protease inhibitor [11–14]. In this study, we used the NS3-4A protease inhibitor telaprevir (VX950; MP424; Mitsubishi Tanabe Pharma Co., Osaka, Japan) [15] and the NS5B RNA polymerase inhibitor MK0608 (2′-C-methyl-7-deaza-adenosine) [16] and investigated the effect of a short term combination treatment with these drugs on HCV replication both *in vitro* and in *vivo*, and showed a successful elimination of viruses in HCV-infected chimeric mice without the use of IFN. Although the dose of the drugs used in this study might be intolerable in humans, elimination of the virus without IFN by only 4 weeks of therapy sheds light on approaches to developing combination therapies using multiple STAT-C agents without IFN.

Materials and methods

Cell culture

An HCV subgenomic replicon plasmid, pRep-Feo, was derived from pRep-Neo (originally, pHC-VIbneo-delS [17]). The pRep-Feo carries a fusion gene comprising firefly luciferase (*Fluc*) and neomycine phosphotransferase, as described elsewhere [18,19]. Replicon RNA was synthesized *in vitro* by T7-RNA polymerase (Promega, Madison, WI) and transfected into Huh7 cells by electroporation. Huh7 cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum at 37 °C under 5% CO_2. After culturing in the presence of G418 (Wako, Osaka, Japan), cell lines stably expressing the replicons were established (Huh7/Rep-Neo).

Luciferase assay

Replicon cell lines were treated with various concentrations of either telaprevir or MK-0608 for 72 hrs, and HCV RNA replication level was quantified by internal luciferase assay. Luciferase activities were quantified using a luminometer (Lumat LB9501; Promega) and the Bright-Glo Luciferase Assay System (Promega). The 50% inhibitory concentrations (IC_{50}) were defined as the drug concentrations producing a 50% reduction in the levels of luciferase activities relative to average levels in untreated cultures.

MTT assays

Cell viability was measured under the same experimental settings using a tetrazolium (MTT)-based viability assay (BioAssay, California, USA) according to the manufacturer's directions. The 50% cytotoxic concentrations (CC_{50}) were defined as the drug concentrations producing a 50% reduction in absorbance relative to the average level in untreated cultures.

Animal treatment

Generation of the $uPA^{+/+}/SCID^{+/+}$ mice and transplantation of human hepatocytes were performed as described recently by our group [20]. All mice were transplanted with frozen human hepatocytes obtained from the same donor. All animal protocols described in this study were performed in accordance with the guidelines of the local committee for animal experiments, and all animals received humane care. Infection, extraction of serum samples, and sacrifice were performed under ether anesthesia. Mouse serum concentrations of human serum albumin (HSA), correlated with the repopulation index [20], were measured as previously described [21]. Eight weeks after hepatocyte transplantation, mice were intravenously injected with 100 μl of HCV-positive human serum samples. Mice serum samples were obtained every one or 2 weeks after HCV infection, and HSA and HCV RNA levels were measured.

Treatment with anti-HCV drugs in HCV-infected mice

Telaprevir and MK-0608 were dissolved with a specific solvent. Eight weeks after HCV infection when the mice developed stable viremia (10^6 to 10^9 copies/ml), mice were administered either 200 mg/kg of telaprevir or 3–50 mg/kg of MK-0608 orally twice a day for 4 weeks. The specific solvent had no anti-HCV effect in this mouse model (data not shown). To analyze the effect of the combination treatment with telaprevir and MK-0608, these drugs were mixed and given together as a cocktail. Human IFN-alpha-treatment was provided daily by intramuscular injection of diluted IFN solution (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) for 4 weeks.

Human serum sample

Human serum containing a high titer of genotype 1b HCV (2.2×10^6 copies/ml) was obtained from a patient with chronic hepatitis who had provided written informed consent to participate in the study. Serum samples were divided into small aliquots and stored in liquid nitrogen until use. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institutional review committee.

RNA extraction and amplification

RNA extraction, nested PCR and quantitation of HCV by real-time polymerase chain reaction (PCR) were performed as described previously [12,13]. Briefly, RNA was extracted from serum samples and extracted livers using SepaGene RVR (Sankojunyaku, Tokyo, Japan) and reverse transcribed with a random hexamer and a reverse transcriptase (ReverTraAce; TOYOBO, Osaka, Japan) according to the instructions provided by the manufacturer. Quantitation of HCV cDNA was performed using Light Cycler (Roche Diagnostic, Japan, Tokyo). The lower detection limit of real-time PCR is 10^3 copies/ml.

Sequence analysis

The nucleotide and amino acid sequences of the NS3 and NS5B region of HCV were determined by direct sequencing following PCR amplification of cDNA after reverse transcription of HCV RNA. The primers used to amplify the NS3 region were 5′-GTGCTCCAAGCTGGCATAAC-3′ and 5′-AGGACCGAGGAATCGAACAT-3′ as the first (outer) primer pair and 5′-CTAGAGTGCCGTACTTCGTG-3′ and 5′-ACTGATCCTGGAGGCGTAGC-3′ as the second (inner) primer pair. The primers used to amplify the NS5B region were 5′-TAAGCGAGGAGGCTGGTGAG-3′ and 5′-CCTATTGGCCTGGAGTGTTT-3′ as the first (outer) primer pair and 5′-GACTCAACGGTCACTGAGAG-3′ and 5′-CCTATTGGCCTGGAGTGTTT-3′ as the second (inner) primer pair. PCR was performed in a 25 μl solution, consisting of a reaction buffer (12.5 μl, 2× PCR buffer for FOD FX), 5 μl 2 mM dNTPs, 0.75 μl F primer (10 μM), 0.75 μl R primer (10 μM), 1 μl Temp DNA (10 pg–200 ng), 0.5 μl KOD FX, 4.5 μl D.W. RT-PCR reactions were carried out following the manufacturer's instructions (Biometra T-Personal; Montreal Biotech Inc., Kirkland, QC, Canada). Amplification conditions included an initial denaturation at 94 °C for 2 min, 35 cycles of amplification (denaturation at 94 °C for 2 min, annealing of primer at 56 °C (1st PCR) or 59 °C (2nd PCR) for 30 s; extension at 68 °C for 2 min 30 s (NS3, 1st PCR), 1 min 30 s (NS3, 2nd PCR), 2 min (NS5B, 1st PCR), or 1 min 10 s (NS5B, 2nd PCR)); and final extension at 68 °C for 5 min.

Results

Anti-viral activity of telaprevir and MK-0608 on HCV subgenomic replicon cells

The effect of telaprevir and MK-0608 on HCV replication was analyzed *in vitro* using HCV replicon cells. Huh7/Rep-Feo cells were treated with various concentrations of either telaprevir or MK-0608. Measured luciferase activity demonstrated that both drugs inhibited HCV replication in a dose-dependent manner (Fig. 1). The IC_{50} of telaprevir and MK-0608 was 0.53 and 0.51 μM, respectively, consistent with previous reports [7,16]. When

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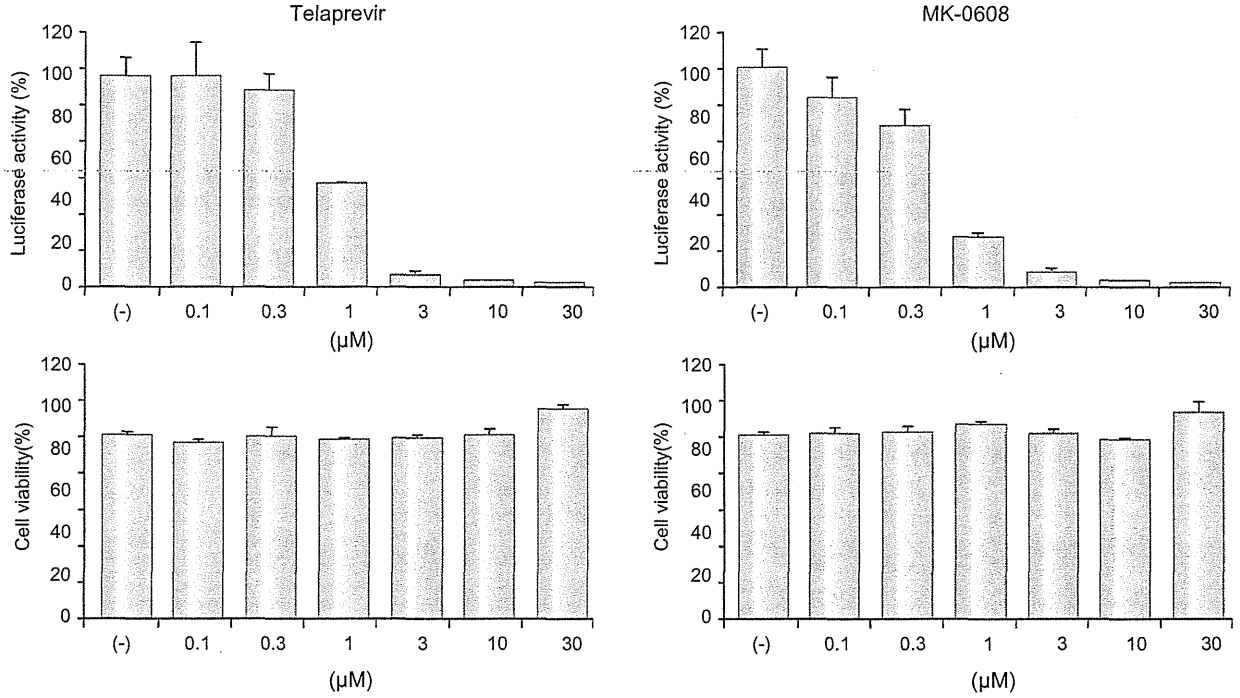

Fig. 1. *In vitro* analysis of susceptibility of HCV replicon cells to anti-HCV drugs. Huh7/Rep-Neo cells were treated for 72 h with the indicated concentrations of either telaprevir or MK-0608. Intracellular HCV RNA replication levels were determined as luciferase activities (upper panel), and expressed relative to cellular viabilities (lower panel). Bars represent means ± SD of three experiments.

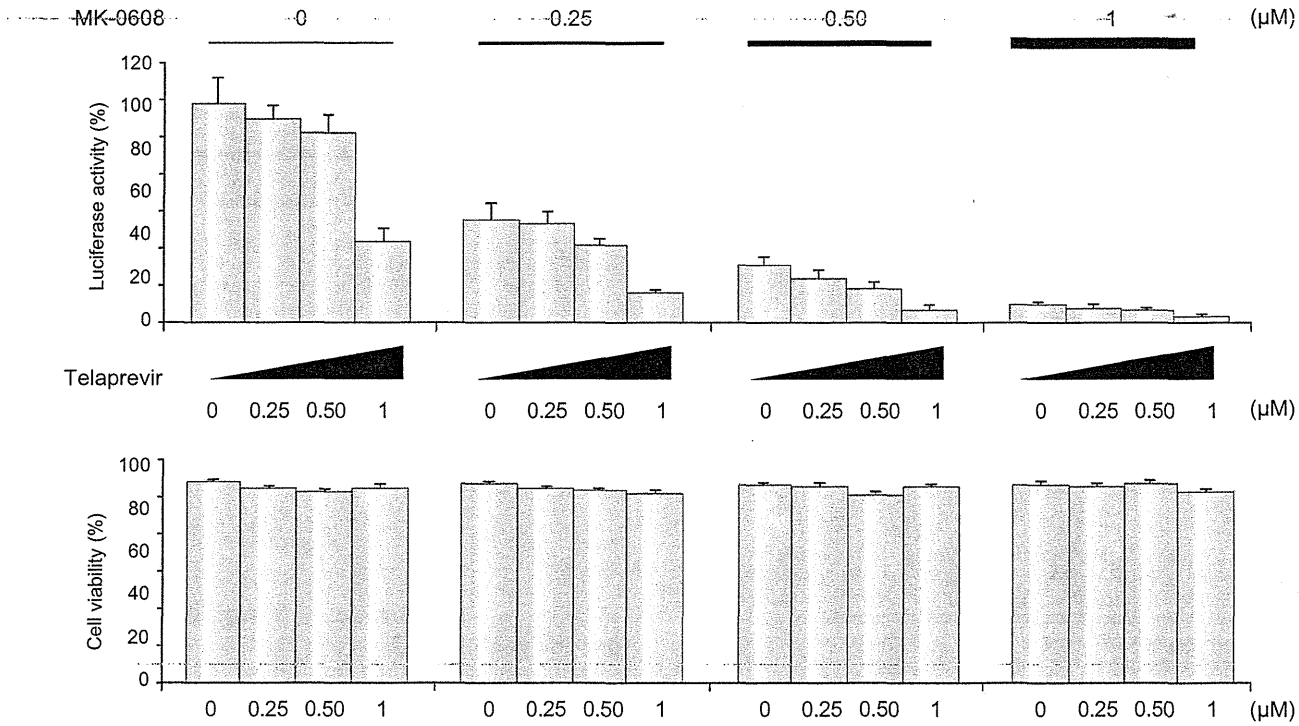

Fig. 2. *In vitro* analysis of susceptibility of HCV replicon cells to combination treatment with anti-HCV drugs. Huh7/Rep-Neo cells were treated for 72 h with the indicated concentration of MK-0608 plus telaprevir. Intracellular HCV RNA replication levels were determined as luciferase activities (upper panel), and expressed relative to cellular viabilities (lower panel). Bars are means ± SD of 3 experiments.

telaprevir and MK-0608 were combined, the anti-HCV effect was increased without cellular damage (Fig. 2).

Effects of telaprevir and MK-0608 on HCV replication in vivo

To analyze the effect of telaprevir and MK-0608 *in vivo*, we used genotype 1b HCV-infected human hepatocyte chimeric mice. Eight HCV-infected mice were treated with either 200 mg/kg of telaprevir or 3 mg/kg of MK-0608 for 4 weeks. At the end of 1 week, treatment resulted in a 1.9 ± 0.7 log reduction of HCV RNA in telaprevir-treated mice and a 2.6 ± 0.2 log reduction in MK-0608-treated mice (Fig. 3A and C). During the treatment, the level of HSA did not decrease. Serum HCV RNA level rebounded in one of the four telaprevir-treated mice and in two of the three MK-0608-treated mice (a MK-0608-treated mouse died after 1 week of treatment). Nucleotide and amino acid sequence analysis showed the emergence of a V36A mutation (NS3-4A protease inhibitor-resistant variant) in the NS3 region (Fig. 3B) in a telaprevir-treated mouse, and a S282T mutation (NS5B polymerase inhibitor-resistant variant) in the NS5B region (Fig. 3D) in MK-0608-treated mice, similar to clinical observations and analysis using HCV-infected chimpanzees [22,23].

Combination treatment with telaprevir and MK-0608 on HCV replication in vivo

Because mono-therapy with either telaprevir or MK0608 resulted in emergence of drug-resistant variants, we analyzed the effect of

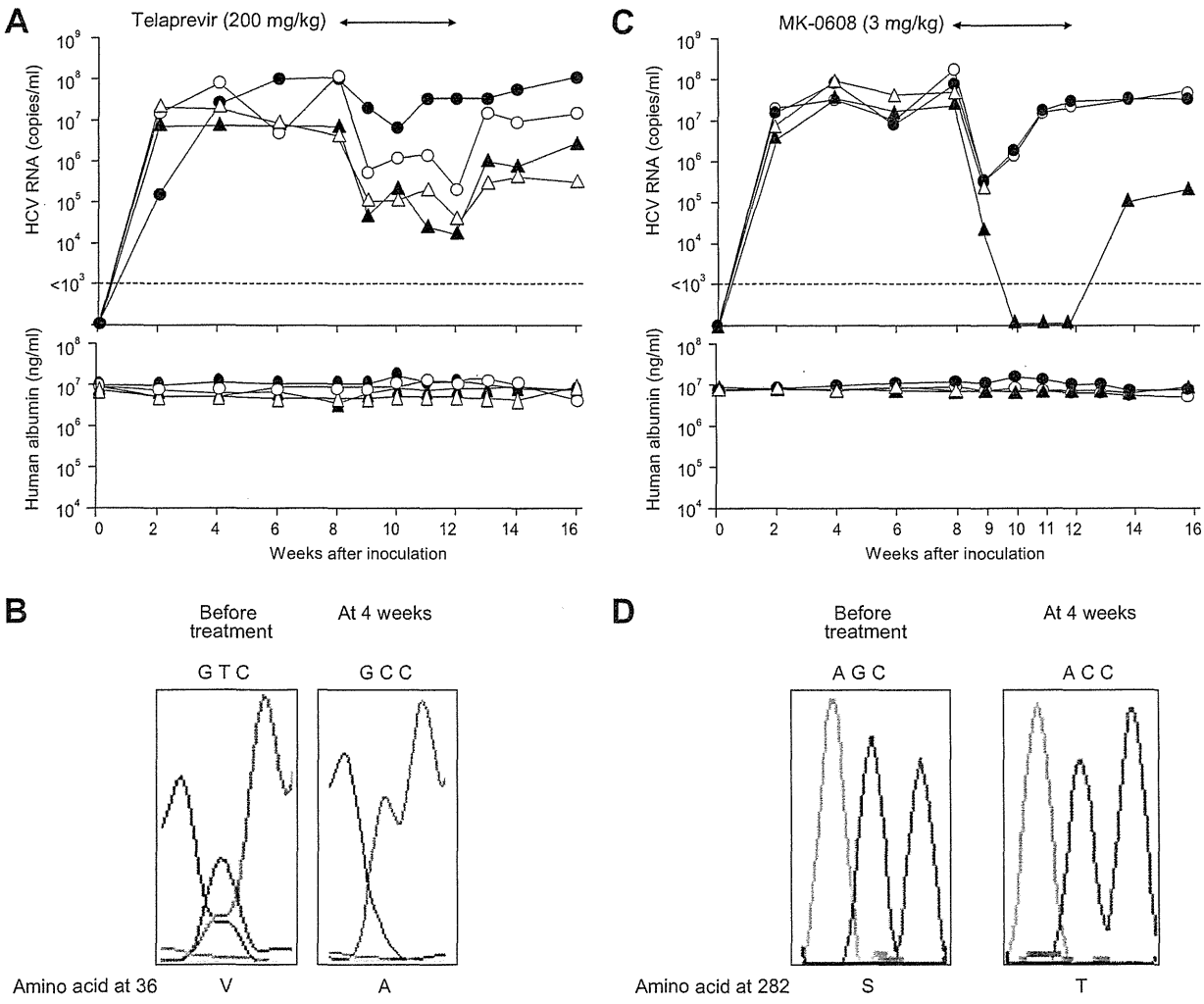

Fig. 3. Antiviral effects of either telaprevir or MK0608 monotherapy on HCV-infected mice. Mice were injected intravenously with 100 μl of HCV-positive human serum samples. Eight weeks after HCV infection, mice were treated with either 200 mg/kg of telaprevir (A) or 3 mg/kg of MK-0608 (C) for 4 weeks. Mice serum samples were obtained at the indicated times, and HCV RNA titer (upper panel) and human serum albumin concentration (lower panel) were analyzed. The horizontal dashed line represents the detection limit (10^3 copies/ml). Note that one telaprevir-treated mouse (A, closed circle) and two MK-0608-treated mice (B, closed circle and open circle) showed a viral breakthrough during the dosing period. Nucleotide and amino acid (aa) sequence analysis of aa 36 in the HCV NS3 (B) or at aa 282 in the NS5B region (D) by direct sequencing in mice serum samples obtained before treatment and at 4 weeks.

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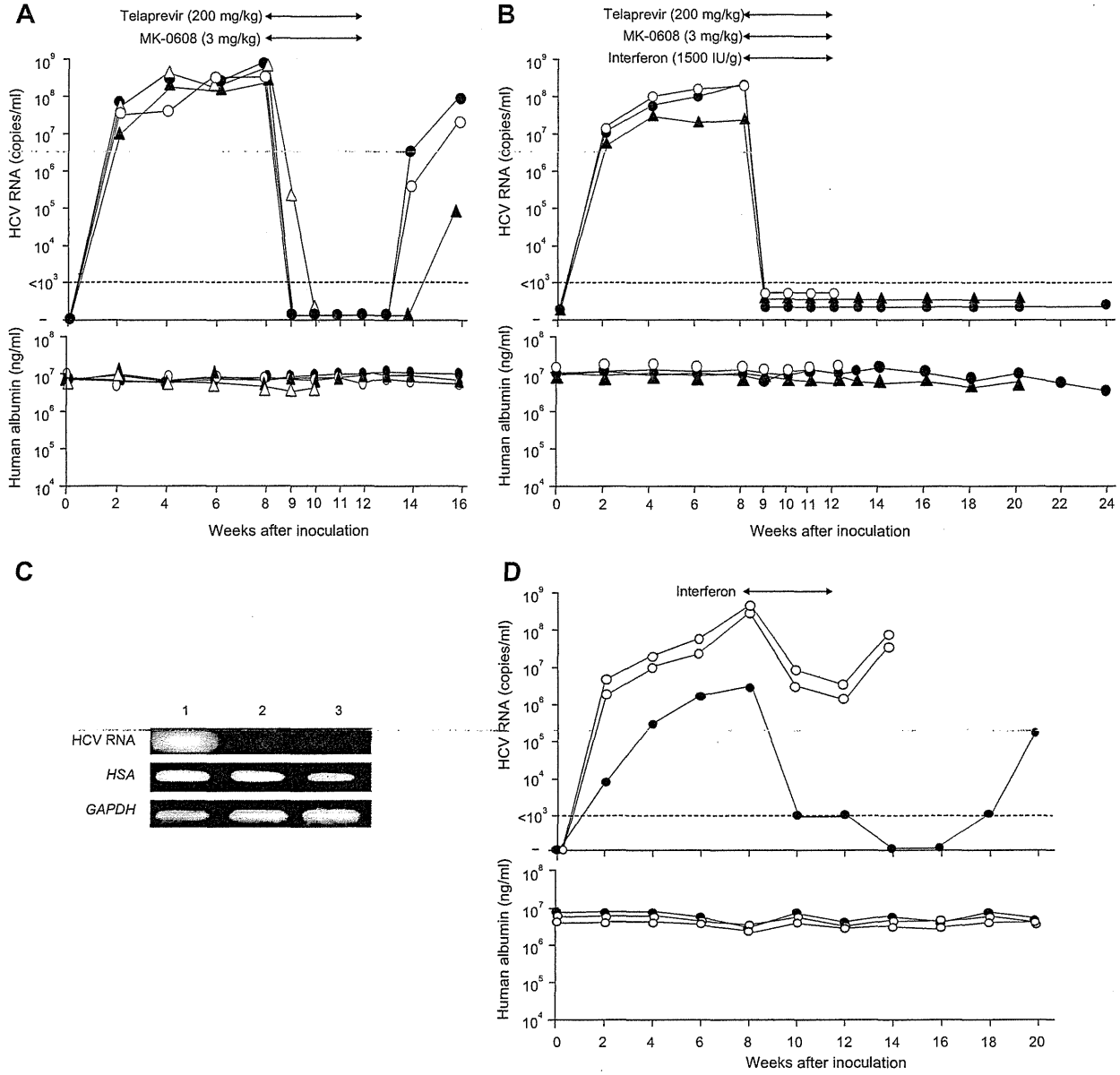

Fig. 4. Antiviral effect of combination treatment on HCV-infected mice. HCV-infected mice were treated with 200 mg/kg of telaprevir plus 3 mg/kg of MK-0608 without (A) or with (B) 1500 IU/g of human interferon-alpha for 4 weeks. Mice serum samples were obtained at the indicated times, and HCV RNA titer (upper panel) and human serum albumin concentration (lower panel) were analyzed. (C) Nested PCR of HCV RNA, human serum albumin (*HSA*) and *GAPDH* in a telaprevir, MK-0608 and interferon-alpha-treated mouse liver at 24 weeks (lane 2). Mice livers with (lane 1) or without (lane 3) HCV-infection were also analyzed. (D) HCV-infected mice were treated with either 1500 (open circles) or 7000 IU/g (closed circles) of interferon-alpha for 4 weeks.

combination treatment of these drugs with or without IFN on HCV replication *in vivo*. Four HCV-infected mice were treated with telaprevir plus MK-0608 for 4 weeks (Fig. 4A). Serum HCV RNA became negative by nested PCR with this combination treatment in all mice. One mouse died after 2 weeks of treatment. During the treatment, no emergence of resistant strains was observed in each of the remaining three mice; however, all mice became positive for HCV RNA again after cessation of the therapy. Another three mice were treated with telaprevir, MK-0608 and IFN-alpha for 4 weeks (Fig. 4B). HCV RNA became undetectable in all three mice 1 week after the beginning of the therapy. After 4 weeks of treatment, one mouse died. In the remaining two mice, HCV RNA did not become positive after cessation of the therapy. One of the remaining two mice died at 20 weeks, and the remaining mouse was sacrificed at 24 weeks (12 weeks after the cessation of therapy). HCV was probably eliminated because no HCV RNA was detected by nested PCR in this mouse liver (Fig. 4C). As a control, HCV-infected mice were treated with 1500 IU/g/day of IFN-alpha alone for 4 weeks, resulting in a two log reduction (Fig. 4D). HCV RNA became undetectable with

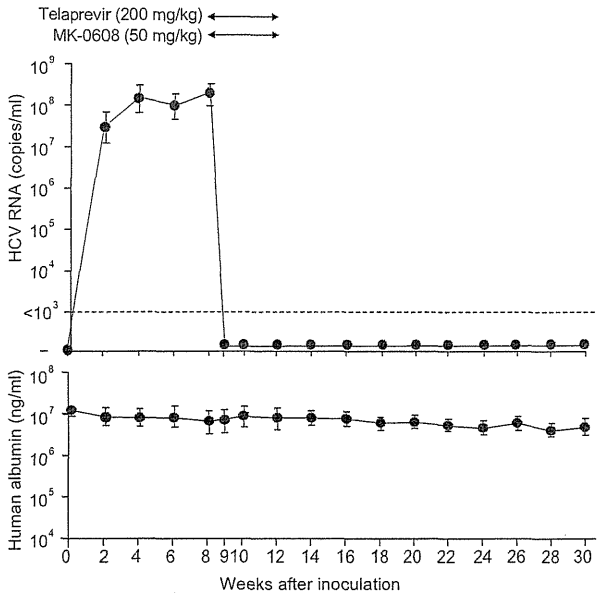

Fig. 5. High doses of MK-0608 and telaprevir combination treatment eliminates virus in HCV-infected mice. *HCV-infected mice were treated with 50 mg/kg of MK-0608 and 200 mg/kg of telaprevir for 4 weeks. Mice serum samples were obtained at the indicated times, and HCV RNA titer (upper panel) and human serum albumin concentration (lower panel) were analyzed. Points represent the means ± SD of five mice.*

administration of 7000 IU/g/day of IFN-alpha treatment. However, the virus rebounded after cessation of the therapy.

Four-week high dose combination therapy of MK-0608 and telaprevir eliminated HCV from mice

We investigated whether combination treatment with high doses of MK-0608 and telaprevir without IFN eliminates viruses from HCV-infected mice. Five HCV-infected mice were treated with high doses of MK-0608 (50 mg/kg) and telaprevir (200 mg/kg) for 4 weeks. Serum HCV RNA titer became undetectable 1 week after commencement of the therapy and remained undetectable in all mice at 30 weeks (18 weeks after cessation of the therapy) (Fig. 5). No apparent toxicity of the drugs was observed as none of the mice showed a decrease in the level of serum HSA.

Discussion

Since we began performing treatment experiments using human hepatocyte chimeric mice with HCV, we have administered many different drugs to analyze the effects on suppression or eradication of the virus. However, until we performed the experiments described in this study, we have never observed long term absence of the virus following cessation of the therapy [12,24]. Strikingly, after only 4 weeks of triple therapy with IFN, telaprevir and MK0608, was long term absence of the virus in mouse serum after cessation of the therapy visible (Fig. 4B). Furthermore, high dose telaprevir and MK-0608 combination therapy resulted in a similar absence of the virus for 16 weeks after cessation of therapy (Fig. 5). In this study, mice were treated with 200 mg/kg of telaprevir twice a day, and 1 week of the treatment resulted in an approximately 2 log reduction of HCV RNA (Fig. 3A), as has been observed previously in chronic hepatitis C patients treated with 450 mg of telaprevir every 8 hrs [25]. This result suggests that approximately 1/15th of a dose in this mouse model may be equivalent to a dose in humans.

During the observation period, some mice died. We do not think that this is due to the drug regimes because the chimeric mouse is weak, and approximately 50% of mice die spontaneously at week 6 after transplantation [26].

Sustained virological response, the complete elimination of the virus from the human body, is defined as testing negative for HCV RNA in serum for more than 24 weeks after cessation of the therapy. As the chimeric mouse used in this study is a weak animal, we were unable to monitor for absence of the virus beyond 24 weeks following cessation of therapy. However, negative testing for HCV RNA in mouse liver by nested PCR (Fig. 4C) 12 weeks after cessation of the therapy strongly suggests that HCV was completely eliminated from the mouse. Of course the mouse model differs from infection in humans where the virus replicates for years in the livers of infected patients. However, results of this study suggest that we will be able to eliminate the virus in humans by treating patients with regimens similar to those used in this study.

Until recently eradication of the virus with biochemical and histological improvement in chronically infected patients has long been reported only with the use of IFN or PEG-IFN [27,28]. Recently, Suzuki et al. reported for the first time eradication of the virus from chronically infected patients without IFN [29].

Elimination of the virus without IFN is desirable due to the many serious side effects of this drug [3,5–9]. However, emergence of drug resistance is a problem, as demonstrated in this study (Fig. 3) as well as in previous studies using replicon systems and HCV-infected chimpanzees [22,23]. A recent clinical study of NS3-4A and NS5B inhibitor combination therapy has reported that 13 days of this combination treatment achieved robust antiviral suppression in chronic hepatitis C patients [30]. As no study has tested the possibility of development of double drug resistant mutants, we will have to test if long term low dose treatment with any combination of STAT-C compounds might induce emergence of multi-drug resistant strains. Furthermore, as there is no report for emergence of IFN resistant strains, regimens such as combination therapy with multiple STAT-C drugs with a small or standard amount of IFN should be tested to develop the best therapy to eradicate the virus with a minimum of side effects and costs. Our further attempts to test possible combinations in mice to determine the best combination of STAT-C drugs will give us an insight into how to develop more effective therapeutic regimens in humans.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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CASE REPORT

A case of hepatitis C-associated osteosclerosis that was improved with the combination therapy of peginterferon alfa-2b and ribavirin

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Abstract Hepatitis C-associated osteosclerosis (HCAO) is a rare disorder characterized by a marked increase in skeletal mass in patients who are infected with hepatitis C virus (HCV). The clinical presentation is an acquired deep bone pain with increased serum alkaline phosphatase (ALP) activity. We present a case of a patient with HCAO who was treated with antiviral therapy. A 42-year-old Japanese man presented with severe, stabbing pain in his lower limbs. He was diagnosed with hepatitis C secondary to intravenous drug use 20 years earlier. Serum biochemical studies revealed markedly elevated ALP activity and osteocalcin levels. Skeletal radiographs showed diffuse bony sclerosis with marked cortical thickening in the long bones. The bony findings and clinical symptoms were attributed to HCAO. The HCV RNA viral load was high and the genotype was 2a. The patient was treated with peginterferon alfa-2b and ribavirin for 24 weeks. After 24 weeks of the combination therapy, the patient had a sustained virological response and clinical remission of bone pain and a decrease in the level of serum ALP. In conclusion, HCAO was improved by the combination therapy of peginterferon alfa-2b and ribavirin when the patient achieved sustained virological response. It was confirmed that HCAO was one of the extrahepatic manifestations of HCV.

Keywords Peginterferon alfa-2b · Ribavirin · Hepatitis C-associated osteosclerosis

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Introduction

Hepatitis C virus (HCV) infection leads not only to liver disease, but also to extrahepatic manifestations [1, 2]. Hepatitis C-associated osteosclerosis (HCAO) is a rare disorder characterized by a marked increase in skeletal mass in patients who are infected with HCV [3–15]. The clinical presentation of such patients is an acquired deep bone pain with increased serum alkaline phosphatase (ALP) activity. However, it is uncertain whether HCV is the causative agent of this skeletal disease. We present a new case of a patient with HCAO who was treated with antiviral therapy, and consider HCAO as an extrahepatic manifestation of HCV.

Case report

A 42-year-old Japanese man was referred to us at Fukuoka University Hospital for severe, stabbing pain in his lower limbs. The pain had begun bilaterally in his knees 6 months previously and had progressed to involve the pre-tibial regions and thighs. There was no history of fracture or skeletal deformity. Serum biochemical studies revealed

markedly elevated bone specific ALP activity and osteocalcin levels, as well as a mild to moderately increased alanine aminotransferase (ALT) level (Table 1). Serum calcium levels were reduced. In urine, deoxypyridinolin (DPD) excretion and crosslinked *N*-telopeptide of type I collagen (NTX) were markedly elevated. Hematology, renal function, serum protein, electrophoresis, and screening for fluorosis as well as heavy metal poisoning were all normal. Family histories were negative. The patient was seropositive for antibody against HCV. The HCV RNA viral load was high and the genotype was 2a. There was a history of intravenous drug abuse 20 years previously. Liver biopsy revealed the histological finding of F2/A2 according to the New Inuyama classification [16]. Skeletal radiographs showed diffuse bony sclerosis with marked cortical thickening in the long bones and with cancellous osteosclerosis in the spine and pelvis (Fig. 1). Bone mineral density of lumbar vertebrae (L2, 3, 4) was 1.991 g/cm^2, approximately twice the mean value for controls. Helical computed tomography imaging showed osteosclerosis with marked cortical thickening along the diaphyses of the bilateral shank (Fig. 2). A 99mTc-methylene diphosphonate bone scan demonstrated diffusely increased radionuclide activity, a finding consistent with markedly increased bone turnover (Fig. 3).

The diagnostic criteria for HCAO have not yet been established. This patient had chronic hepatitis C and osteosclerosis with severe bone pain. Although markers of bone formation such as serum ALP activity and osteocalcine were invariably high, markers of bone resorption such as urinary DPD and NTX were also increased. The radiographic finding indicated osteosclerosis as a result of bone formation and bone resorption. Furthermore, there were no diseases causative of osteosclerosis such as fractures, skeletal deformity or bone tumor. The patient was diagnosed with hepatitis C secondary to intravenous drug use, and the bony findings and clinical symptoms were attributed to HCAO.

Although the patient received non-steroidal anti-inflammatory drugs (NSAIDs) for the alleviation of bone pain, his bone pain was not improved. The patient was treated with peginterferon alfa-2b (PegIntron, Schering-Plough) subcutaneously once weekly at a dose of 100 μg (1.5 μg/kg) and ribavirin (Rebetol, Schering-Plough) was administered orally at a dose of 800 mg/day. This combination therapy was continued for 24 weeks. Clinical biochemical laboratory values and serum HCV RNA were assessed before therapy, at 4-week intervals throughout the 24-week treatment period and during a post-treatment follow-up period. After the 4 weeks of the combination therapy, ALT was normalized and serum HCV RNA was negative. A serum bone specific ALP level was markedly decreased. Because the level of serum calcium in the patient was decreased after the combination therapy, calcium at a dose of 3–6 g/day was administrated orally to the patient (Fig. 4). The combination therapy of peginterferon alfa-2b and ribavirin for 24 weeks obtained sustained virological response in the patient. After 24 weeks of the combination therapy, the patient had a clinical remission of bone pain and a decrease in the level of serum bone specific ALP. Because the patient had no bone pain after the combination therapy, the use of NSAIDs was not necessary. Table 2 shows the change in laboratory data. After the HCV clearance, bone mineral metabolisms were improved.

We recommended a computed tomography or bone scintigram to the patient for determining whether bone hypertrophy was improved. However, the patient has not accepted more radiological examination. Because we were not able to get the informed consent of our patient, we could not present the radiological change after the combination therapy of peginterferon alfa-2b and ribavirin.

Discussion

The bone pain of the patient with HCAO improved when the patient attained a sustained virological response with the combination therapy of peginterferon and ribavirin. This is the first report to prove that HCAO is an extrahepatic manifestation of HCV.

In 1990, Beyer et al. [3] reported a case of idiopathic acquired diffuse osteosclerosis. Thereafter, several cases of painful diffuse osteosclerosis after drug abuse were reported [4, 5, 7]. Patients were found to be seropositive for HCV. In 1997, Whyte and Reasner [8] described this new skeletal disorder as HCAO. HCAO is characterized by a generalized increase in bone mass in adults who are infected with HCV. These patients have a deep, mild or severe pain and tenderness of the limbs, with no record of previous fractures. Radiographs show diffuse bone sclerosis with marked cortical thickening. Bone scintigraphy shows a diffuse increased uptake of radionuclide; however bone biopsy shows normal lamellar bone with accelerated rates of skeletal formation [9, 14]. Although markers of bone formation such as serum ALP activity and osteocalcine are invariably high, markers of bone resorption such as urinary DPD and NTX are also increased. Furthermore, areal bone mineral density is abnormally high. However, HCAO is thought to be rare among HCV seropositive patients, because a study looking at the skeletal radiographs of 107 randomly selected HCV patients failed to demonstrate dense bones in these patients [17].

This patient had the clinical hallmarks of HCAO. However, hypocalcemia was found and serum PTH level along with 1,25-hydroxyvitamin D level were increased. We