TABLE 3. Primers

Gene	Forward primers (5′–3′)	Reverse primers (5′–3′)
IGF-I	GCTTCCGGAGCTGTGATCTAA	GCTGACTTGGCAGGCTTG
SOCS2	GCAAGGATAAGCGGACAGG	GCGGTTTGGTCAGATAAAGGT
NNMT	CCGGGAGGCAGTAGAGGC	GTCCTTCGTTGTTGGCCAT
IGFLS	TCTGCAGGGCGAAGTCC	
KLOTHO	AGCCATTATACCACCATCCTTG	GTCGGTCATTTCTTGCACTTCTA
P4AH1	TGGATACCCATTTGTTGCCA	
SLC16A1	TGCTGGAGCCCTCATGC	TTCCAGCTTTCTCAAGGGATG
SRD5A1	TACGTATTCAAATAAGCCTCCCCT	
SCD	TCAAAACAGTGTGTTCGTTGC	CATAAGGACGATATCCGAAGAGG
FADS1	CAGGCCACATGCAATGTC	ATCTAGCCAGAGCTGCCCTG
FADS2	GGCTCTCCAGGAACCTGATG	
FASN	GGCAAATTCGACCTTTCTCAG	AGGACCCCGTGGAATGTC
DGAT2	ACGGCCTTACCTGGCTACA	AGACATCAGGTACTCCCTCAACAC
ADPN	CCTCCAGGTCCCAAATGCC	CCAGTCCTGCTCAGGTGTGC
AKR1B10	GGCCTGTAACGTGTTGC	ATGGGACATGAGTGGAGG
SREBP1c	CATGGATTGCACATTTGAAG	CAGAGAGGAGGCCAGAGAA
FABP	GATCCAAAACGAATTCACGG	ATTGTCACCTTCCAACTGAACC
GAPDH	CCACCTTTGACGACGCTGGG	CATACCAGGAAATGAGCTTGACA

synthesized using 1 μg RNA and PowerScript reverse transcriptase (Clontech, Mountain View, CA) and oligo-deoxythymidine primers (Invitrogen) and was subjected to real-time qRT-PCR following the manufacturer's instructions. Genes were amplified with a set of gene-specific primers (Table 3) and SYBR Green PCR mix in a PRISM 7700 sequence detector (Applied Biosystems, Tokyo, Japan). These primers were capable of amplifying human, but not mouse, genes. PCR products were monitored during amplification. All data were calculated by the comparative threshold cycle (Ct) method (22). Occupancy rates of h-hepatocytes in h-hepatocyte regions ranged from 70–95% (Table 2). Contamination of m-hepatocytes did not affect RT-PCR results of human gene expression because each gene's expression level was normalized against h-*GAPDH*.

Responsiveness of h-hepatocytes$_{\text{chimeric mouse}}$ to h-GH and h-IGF-I

Hepatocytes synthesize and secrete IGF-I when GH receptors are activated by GH (23). To determine whether h-GH directly regulates GH-responsive genes or h-IGF-I, h-hepatocytes$_{6YF}$ (9×10^5 cells) from three chimeric mice were cultured in 1.8-cm Matrigel-coated dishes in DMEM as previously reported (24), and 4 h later, they were exposed with 0, 5, and 50 ng/ml h-GH or 50 and 500 ng/ml h-IGF-I for an additional 24 and 48 h and harvested in RLT buffer to prepare total RNA for real-time qRT-PCR.

Gene enrichment analysis

Gene and gene ontology (GO) information were collected from NCBI build 37.1 (ftp://ftp.ncbi.nlm.nih.gov/gene/DATA/gene2go.gz) and The Gene Ontology (http://www.geneontology.org/ontology/gene_ontology.obo) sites, respectively. Pathway information was collected from KEGG (ftp://ftp.genome.jp/pub/kegg/pathway/organisms/hsa/hsa_gene_map.tab) and Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems, Redwood City, CA). The gene enrichment analysis was performed using only GO and pathway groups where at least two genes or more were assigned.

Statistics

Microarray data were evaluated by the Welch's *t* test (two-sided). The gene enrichment analysis was calculated using Fisher's exact test and corrected with Benjamini-Hochberg's false discovery rate (25). The significance of overlap between two groups of transcripts was determined using Fisher's exact test. Log_{10}-transformed data obtained in real-time qRT-PCR analysis of *in vivo* and *in vitro* studies were analyzed among groups by ANOVA. When the overall F statistics were significant, significance was determined by the Scheffé's test with significance level $\alpha = 0.05$.

Results

Lipid accumulation in chimeric mouse livers

Hepatocytes$_{6YF}$ were transplanted to uPA/SCID mice, and the process of h-hepatocyte repopulation in host livers was visualized using hematoxylin- and eosin-stained histological sections. Vacuoles appeared in the cytoplasm of donor h-hepatocytes approximately 70 d after transplantation and gradually increased in numbers and sizes thereafter (Fig. 1A, a and b). To test whether these vacuoles represent lipid deposits, 36 chimeric mice$_{6YF}$ were killed 48–111 d after transplantation (five before 60 d, 28 between 70 and 90 d, and three after 90 d) for ORO staining of liver sections (Fig. 1B). Most of the chimeric liver h-hepatocytes became ORO$^+$ approximately 70 d after transplantation. The steatosis level was quantified by the size and frequency of ORO$^+$ lipid droplets from grade 0 (Fig. 1Ba) to grade 3 (Fig. 1Bd) and plotted against post-transplantation days (Fig. 1C). Among five livers before 60 d of transplantation, one and four livers were of grade 0 and 1, respectively (Fig. 1C). Most of the livers between 70 and 90 d were of grade 1 (11 of 28 mice) and grade 2

(nine of 28). All three livers after 90 d were of grade 3, showing a good correlation of the steatosis level with post-transplantation duration (~50–110 d). h-IGF-I serum levels of chimeric mice$_{6YF}$, a measure of h-GH level, were under a detection limit of 9.4 ng/ml (n = 3), which supported our previous study that chimeric mouse h-hepatocytes were h-GH deficient (6). Therefore, we considered h-GH deficiency as an etiological factor in the observed hepatic lipogenesis.

Improvement of liver steatosis in chimeric mice by h-GH

To examine the relationship of steatosis with-GH-deficiency, five chimeric mice$_{6YF}$ at different time points after transplantation were infused with h-GH during the last 2 wk before killing (one mouse was killed at 83 d, three at 84 d, and the remaining one at 89 d) and were used as h-GH-treated chimeric mice. Twenty-eight of 36 chimeric mice$_{6YF}$ that were used in the experiment shown in Fig. 1C and killed 70–90 d after transplantation served as controls. The h-IGF-I serum level rose to 72.5 ± 9.4 ng/ml (n = 3) in h-GH-treated mice, a level comparable to that in normal human sera, proving the effectiveness of the h-GH treatment. Serial histological sections were immunostained for h-CK8/18 to identify h-hepatocytes (Fig. 2, A and B) and stained with ORO (Fig. 2, C and D). ORO$^+$ droplets were present in the control mouse h-hepatocytes (Fig. 2, A and C) but were not in h-GH-treated ones (Fig. 2, B and D). Some host m-hepatocytes also contained small cytoplasmic ORO$^+$ droplets (Fig. 2, A and C), probably due to uPA damage because even after h-GH treatment, these lipid droplets remained (Fig. 2, B and D). Steatosis grading on liver sections showed that most of the control mouse livers (93%) were of grade 1–3: 39, 32, and 21% for grades 1, 2, and 3, respectively (Fig. 2E). All h-GH-treated livers were of grade 0. Therefore, we concluded that h-GH plays a critical role in the etiology of human liver steatosis.

h-GH-induced changes in gene expression profiles at the hepatocyte level

Hepatocytes were isolated from three h-GH-untreated chimeric mice$_{9MM}$ 72–101 d after transplantation (nos. 1–3) and three h-GH-treated chimeric mice$_{9MM}$ 75 d after transplantation (nos. 4–6) for microarray analysis (Table 2). We found 15,826 positive transcripts (29%) in 54,675 spotted transcripts in either h-GH-untreated or -treated h-hepatocytes$_{\text{chimeric liver}}$. Among these, 229 (1.4%) and 269 (1.7%) transcripts showed more than 2-fold higher and lower expression levels in h-GH-treated than -untreated h-hepatocytes, respectively. Statistical evaluation at $P < 0.05$ selected 58 genes (82 transcripts) from 229 transcripts as up-regulated in h-GH-treated h-hepatocytes$_{\text{chimeric mouse}}$. Similarly, 33 genes (37 transcripts) were selected from the 269 transcripts as down-regulated genes.

Gene enrichment analysis on transcripts showing more than 2-fold changes selected the significantly overrepresented (GH-induced and -suppressed) GO terms and pathways including GH signaling, IGF-I receptor binding, response to hormone stimulus, lipid biosynthetic process, and aging (Table 4 and Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

TABLE 4. Extracted significantly overrepresented GO terms and pathways

Pathway, or GO term	*P* value	B-H FDR q-value
Hepatocyte level		
Pathway		
GH signaling	0.000244	0.00830
GO molecular function		
IGF receptor binding	3.77×10^{-5}	0.00464
GO biological process		
Response to hormone stimulus	4.30×10^{-5}	0.00556
Lipid biosynthetic process	0.000898	0.0251
Lipid metabolic process	0.00122	0.0284
Aging	0.00288	0.0334
Regulation of fatty acid biosynthetic process	0.00353	0.0358
Regulation of lipid metabolic process	0.0241	0.0947
Tissue level		
Pathway		
Biosynthesis of unsaturated fatty acids	2.78×10^{-5}	0.000584
GH signaling	0.0116	0.0612
GO molecular function		
Stearoyl-coenzyme A 9-desaturase activity	8.78×10^{-5}	0.00382
IGF receptor binding	0.00256	0.0500
GO biological process		
Fatty acid metabolic process	0.000189	0.00585
Lipid metabolic process	0.000739	0.0133
Oxidation reduction	0.00312	0.0288
Response to hormone stimulus	0.00468	0.0346
Aging	0.00627	0.0392
Unsaturated fatty acid biosynthetic process	0.0186	0.0692

B-H FDR, Benjamini-Hochberg's false discovery rate.

h-GH-induced changes in gene expression profiles at the liver tissue level

Identical microarray analysis was performed at the liver tissue level with six chimeric mice$_{6YF}$ (Table 2), with half (nos. 1–3) being used as controls and the other half (nos. 4–6) as h-GH-treated mice. In this analysis, h-hepatocyte-repopulated regions were dissected from liver tissues of these animals and used as h-liver$_{\text{chimeric mouse}}$ as RNA sources for microarray analysis in which 54,675 transcripts were spotted as in the case of the hepatocyte-level analysis. Transcripts positive for either h-GH-untreated or -treated h-liver$_{\text{chimeric mouse}}$ were 18,210 (33%) transcripts, among which 146 (0.8%) and 237 (1.3%) transcripts were expressed at more than 2-fold higher and lower levels, respectively, in h-GH-treated tissues than in h-GH-untreated controls. Through statistical evaluation ($P < 0.05$), we identified 43 genes (64 transcripts) and 55 genes (76 transcripts) as up- and down-regulated genes by h-GH from the 146 and 237 transcripts, respectively.

Gene enrichment analysis on transcripts showing more than 2-fold changes selected the significantly overrepresented (GH-induced and -suppressed) GO terms and pathways including biosynthesis of unsaturated fatty acids, GH signaling, stearoyl-coenzyme A desaturase (SCD) activity, IGF receptor binding, oxidoreductase activity, fatty acid metabolic process, aging were significantly changed (Table 4 and Supplemental Table 2).

In summary, we selected 58 up-regulated and 33 down-regulated genes from the h-hepatocyte-level assay and 43 up-regulated and 55 down-regulated genes from the h-liver tissue-level assay. From them, we chose genes that were commonly up- and down-regulated at both the hepatocyte and liver tissue levels. As a result, 14 up-regulated genes (23 transcripts) and four down-regulated genes (five transcripts) were finally identified as more reliable candidates for h-GH-responsive genes as listed in Table 5, in which the expression ratios at the hepatocyte level [h-GH-treated h-hepatocytes$_{\text{chimeric mouse}}$ *vs.* h-GH-untreated h-

TABLE 5. h-GH-regulated genes

Affymetrix ID	Gene symbol	Accession Number	Gene name	Cell level, treated/untreated		Tissue level, treated/untreated	
				Microarray	RT-PCR[a]	Microarray	RT-PCR[a]
Up-regulated							
209988_s_at	*ASCL1*	NM_004316.3	Achaete-scute complex-like 1	123.39	–	151.42	–
209540_at	*IGF1*[b]	AU144912	IGF-I	179.66	159.83	34.19	35.45
203373_at	*SOCS2*[b]	NM_003877	Suppressor of cytokine signaling 2	39.01	73.79	13.91	53.20
202237_at	*NNMT*[b]	NM_006169	Nicotinamide *N*-methyltransferase	46.02	40.09	14.28	20.79
205978_at	*KL*[b]	NM_004795	Klotho	39.90	22.50	6.58	8.45
207543_s_at	*P4HA1*[b]	NM_000917	Procollagen-proline, 2-oxoglutarate 4-dioxygenase, α-polypeptide I	15.17	11.99	9.69	9.03
203498_at	*DSCR1L1*	NM_005822	Down syndrome critical region gene 1-like 1	5.77	–	4.06	–
215712_s_at	*IGFALS*[b]	AW338791	IGF-binding protein, acid labile subunit	5.29	9.49	7.29	13.63
209967_s_at	*CREM*	D14826	cAMP-responsive element modulator	4.41	–	2.76	–
207256_at	*MBL2*	NM_000242	Mannose-binding lectin 2, soluble	3.44	–	2.09	–
222108_at	*AMIGO2*	AC004010	Adhesion molecule with Ig-like domain 2	2.89	–	2.84	–
201309_x_at	*C5orf13*	U36189	Chromosome 5 open reading frame 13	2.75	–	3.47	–
202234_s_at	*SLC16A1*[b]	BF511091	Solute carrier family 16, member 1	2.74	2.29	4.14	3.84
211056_s_at	*SRD5A1*[b]	BC006373	Steroid-5-α-reductase, α-polypeptide 1	2.29	1.99	2.03	1.72
Down-regulated							
208964_s_at	*FADS1*[b]	AL512760	Fatty acid desaturase 1	0.43	0.51	0.36	0.29
219295_s_at	*PCOLCE2*	NM_013363	Procollagen C-endopeptidase enhancer 2	0.37	–	0.45	–
206561_s_at	*AKR1B10*[b]	NM_020299	Aldo-keto reductase family 1, member B10	0.22	0.22	0.19	0.13
202628_s_at	*SERPINE1*	NM_000602	Serine proteinase inhibitor, clade E, member 1	0.17	–	0.28	–

Treated indicates h-GH-treated chimeric mouse, whereas untreated indicates h-GH-untreated chimeric mouse. –, Not determined.

[a] The expression level of each gene was divided with that of h-*GAPDH*.

[b] Gene expression levels were determined by both microarray assay and real-time qRT-PCR.

hepatocytes$_{\text{chimeric mouse}}$ (cell level, treated/untreated)] and at the tissue level [h-GH-treated h-liver$_{\text{chimeric mouse}}$ *vs.* -untreated h-liver$_{\text{chimeric mouse}}$ (tissue level, treated/untreated)] are presented for each gene. *P* values for overrepresentation of the overlapping genes (up- and down-regulated genes in both hepatocytes and liver tissue levels) were 5.34×10^{-9} and 1.92×10^{-9}, respectively, which indicates the significance of the overlapping.

The microarray assay's results were validated by real-time qRT-PCR using RNA extracted from the sources shown in Table 2 on arbitrarily selected eight and three genes from the above final 14 h-GH-up- and four h-GH-down-regulated genes, respectively: *IGF-I*, suppressor of cytokine signaling 2 (*SOCS2*), nicotinamide *N*-methyltransferase (*NNMT*), *KL*, *P4HA1*, *IGFALS*, solute carrier family 16/member 1 (*SLC16A1*), and steroid-5-α-reductase and α-polypeptide 1 (*SRD5A1*) as h-GH-up-regulated genes and fatty acid desaturase (*FADS1*) and aldo-keto reductase family 1/member B10 (*AKR1B10*) as h-GH-down-regulated genes. The expression ratios (treated/untreated) calculated from the qRT-PCR results are included in Table 5, which well support the microarray data, indicating the reliability of the microarray data.

There was the possibility that mouse transcripts were also included as the cDNAs hybridized in the currently adopted microarray assay. To check this possibility, pooled cDNAs of three uPA/SCID mouse livers were subjected to the microarray with 54,675 cDNA spots, which gave a result that 5,643 of 54,675 transcripts (10.3%) were positive. Sixteen genes among the genes listed in Table 5 were not found in these positive genes, but two genes, *SOCS2* and *IGFALS*, both h-GH-up-regulated genes, were found there. Considering that the cross-hybridized signals were less than 10% of those in the GH-untreated hepatocytes$_{\text{chimeric mouse}}$ and the contamination of mouse hepatocytes in the h-hepatocyte preparation used in the present study was less than 10% (Table 2, 9MM nos. 1–6), we concluded that their ratios of treated to untreated genes were high enough to include them as h-GH-regulated genes in the present study. This conclusion was further validated by measuring m-Alb mRNA expression levels in the h-liver$_{\text{chimeric mouse}}$. Real-time qRT-PCR was performed for RNAs isolated from h-liver$_{\text{chimeric mouse}}$ (Table 2, 6YF nos. 1–6) using a set of m-Alb primers. The result showed that m-Alb expression levels in the h-liver$_{\text{chimeric mouse}}$ were 0.5 ± 0.2% of those of the uPA/SCID mouse liver. As a whole, it can be said that the cross-reactivity does not affect the results in the present study.

Improvement of gene expression by h-GH

Real-time qRT-PCR was performed for livers of h-GH-untreated chimeric, h-GH-treated chimeric mice, and humans, the last of which accurately reflect the physiology of h-GH endocrine regulation. The h-GH-untreated and -treated h-hepatocytes$_{\text{chimeric mouse}}$ were isolated from nos. 1–3 and nos. 4–6 of chimeric mice$_{\text{9MM}}$, respectively (Table 2). The h-GH-untreated and -treated h-liver$_{\text{chimeric mouse}}$ were isolated from nos. 1–3 and nos. 4–6 of chimeric mice$_{\text{6YF}}$, respectively (Table 2). The h-hepatocytes$_{\text{human}}$ and h-liver$_{\text{human}}$ were isolated from four (28YM, 57YM, 25YF, and 61YF) and three (28YM, 25YF, and 61YF) donors, respectively (Table 1). Expression levels in h-hepatocytes$_{\text{chimeric mouse}}$ and h-liver$_{\text{chimeric mouse}}$ under h-GH-untreated and -treated conditions were divided by the h-hepatocyte$_{\text{human}}$ and the h-liver$_{\text{human}}$, respectively, which is shown as the hepatocyte ratio (*white bars*) and liver-tissue ratio (*black bars*), respectively (Fig. 3). The ratios are used as measures of the extent of difference/closeness of the gene expression level in h-GH-treated or -untreated chimeric livers from/to that in human liver. If h-GH improves gene expressions in chimeric mouse livers, ratios for h-GH-up-regulated genes in h-GH-untreated and -treated chimeric liver are expected to be less than 1 and approximately 1, respectively, and ratios for h-GH-down-regulated genes in h-GH-untreated and -treated chimeric liver are expected to be more than 1 and approximately 1, respectively.

Expression levels of a total of eight h-GH-up-regulated genes were compared between h-GH-untreated and -treated chimeric mice at both hepatocyte and liver tissue levels. The results of the h-GH-up-regulated genes are shown in Fig. 3A. Generally, the expressions of the genes, except *KL*, were significantly suppressed in the absence of h-GH at both the hepatocyte and liver tissue levels. Expression in h-GH-treated cases was similar to that in human livers: *IGF-I* and *P4AH1* at the tissue level and *IGFALS* and *SLC16A1* at both levels. *KL* expression in h-GH-untreated chimeric mice was similar to that in human livers at both levels, and h-GH treatment markedly increased expression over that in human livers at both levels, suggesting that its expression is greatly up-regulated by GH. *In vivo*, h-GH-up-regulated genes of human livers are likely positively induced by GH.

Our results on suppression of spontaneous lipogenesis by GH (Fig. 2) and a reported relationship between GH-responsive genes and lipogenesis-related genes (26) suggest an association between the h-GH-responsive genes listed in Table 5 and the observed hepatic lipogenesis. Gene enrichment analysis showed that h-GH-responsive genes were enriched as those involved in the lipid synthesis process, lipid metabolic process, and regulation of fatty acid biosynthetic process (Table 4). Of the two down-regulated genes, *FADS1* is known to be lipogenesis related (27), and *AKR1B10* was recently reported to regulate fatty acid synthesis (28). Five genes were additionally cho-

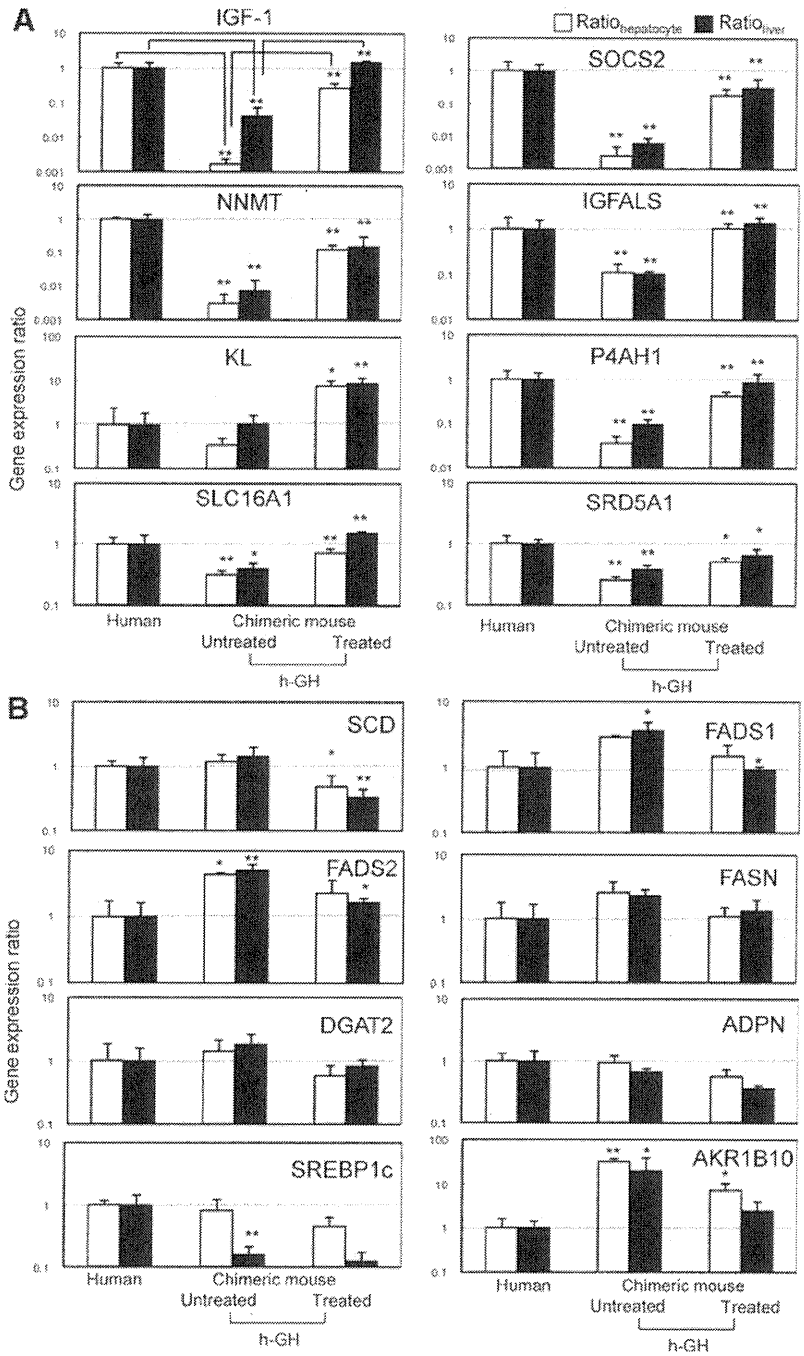

FIG. 3. Regulation of gene expression in h-hepatocytes$_{chimeric\ mouse}$ by h-GH at the hepatocyte and liver tissue levels. Six chimeric mice$_{9MM}$ and six chimeric mice$_{6YF}$ were produced (Table 2); half of each group (nos. 1–3 for both the chimeric mice$_{9MM}$ and chimeric mice$_{6YF}$) served as control animals, and the remaining half (nos. 4–6 for both the chimeric mice$_{9MM}$ and chimeric mice$_{6YF}$) were treated with h-GH. h-Hepatocytes$_{chimeric\ mouse}$ and h-livers$_{chimeric\ mouse}$ were isolated from the former and latter chimeric mice, respectively. h-Hepatocytes$_{human}$ and h-livers$_{human}$ were also isolated from four (25YF, 28YM, 57YM, and 61YF) and three (25YF, 28YM and 61YF) human donors, respectively (Table 1). RNA was isolated from the hepatocytes and liver tissue for real-time qRT-PCR analysis. qRT-PCR was performed for eight h-GH-up-regulated (A) and eight lipogenesis-related (B) genes. The expression level of each gene was normalized against that of h-*GAPDH*. The expression level of h-GH-untreated h-hepatocytes$_{chimeric\ mouse}$ and h-GH-treated h-hepatocytes$_{chimeric\ mouse}$ was divided by that of h-hepatocytes$_{human}$ (ratio$_{hepatocyte}$). Similarly, the expression level of h-livers$_{chimeric\ mouse}$ was divided by that of h-livers$_{human}$ (ratio$_{liver}$). *White* and *black bars* represent the ratio$_{hepatocyte}$ and the ratio$_{liver}$, respectively. Each value represents the mean ± SD. *Asterisks above bars* of untreated chimeric mouse show significance between human and GH-untreated chimeric mouse. *Asterisks above bars* of treated chimeric mouse show significance between human and GH-treated chimeric mouse. *, $P < 0.05$; **, $P < 0.01$.

sen to examine the relationship between h-GH-down-regulated genes and known lipogenic genes from previous studies: *FADS2* (27), *SCD* (29), *FASN* (30), diacylglycerol acyltransferase 2 gene (*DGAT2*) (31), and the adiponutrin gene [*ADPN* (32), currently known as *PNPLA3* (33)]. These genes were included as h-GH-down-regulated genes at either the hepatocyte or liver tissue level in the microarray assay; *FADS2* and *SCD* were significantly ($P < 0.05$) down-regulated only at the liver tissue level, *FASN* was insignificantly (>2-fold) down-regulated only at the hepatocyte level, *DGAT2* was insignificantly (>2-fold) down-regulated only at the hepatocyte level, and *ADPN* significantly ($P < 0.05$) decreased its expression only at the hepatocyte level. Two known GH-inducible lipogenesis-related genes, *SREBP1c* (34–36) and fatty acid-binding protein gene (*FABP*) (24, 37), were also chosen from previous studies.

Expression of a total of nine genes was compared between human livers and h-GH-untreated and -treated chimeric mice at both hepatocyte and liver tissue levels as above. Results for lipogenesis-related genes are shown in Fig. 3B. Ratios of three genes, *FADS1* (significant at tissue level), *FADS2* (significant at both levels), and *AKR1B10* (significant at both levels), were higher in the h-GH-untreated chimeric mice compared with humans, and h-GH treatment lowered the ratios of *SCD* (significant at both levels), *FADS1* (significant at tissue level), *FADS2* (significant at tissue level), and *AKR1B10* (significant at hepatocyte level). Although not significantly, ratios of *FASN* and *DGAT2* were also higher in the h-GH-untreated chimeric mice compared with humans and decreased by h-GH treatment. *ADPN* expression in h-GH-untreated chimeric mice was close to that in humans at both levels, and h-GH treatment decreased ratios (insignificant). Thus, it is most likely that these lipogenesis-related genes are down-regulated by h-GH. Ratios of *SREBP1c* (Fig.

3B) and *FABP* (data not shown) genes did not show any meaningful changes by h-GH at either the hepatocyte or liver tissue level under the observed endocrinological conditions, although *SREBP1* expression was significantly lower at tissue levels in h-GH-untreated chimeric mice compared with human.

Deletion of GH receptor gene (*GHR*) in mice resulted in an increase of insulin receptor gene (*IRS*) expression (38) and a reduction of plasma levels of IGF-I, insulin, and glucose, implying that the mice increased insulin sensitivity (39, 40). These studies suggested the possibility that chimeric mice are insulin sensitive. Thus, we examined whether chimeric mice are insulin sensitive by determining the expression levels of h-*GHR* and h-*IRS*. Real-time qRT-PCR analysis showed that h-*GHR* and h-*IRS* expression levels in chimeric mice were similar or higher than humans, and h-GH administration of chimeric mice did not affect these observed expression levels. However chimeric mice did not show any sign of insulin resistance or sensitivity in a sugar tolerance test (data not shown). As a whole, we currently consider that chimeric mice are not insulin sensitive.

In vitro effects of h-GH on gene expressions in h-hepatocytes

We asked whether the aforementioned effects of h-GH on the h-GH-up-regulated gene and lipogenic gene expression in chimeric mouse livers *in vivo* are reproducible *in vitro*. h-Hepatocytes$_{6YF}$ isolated from three chimeric mice with RI_{Alb} higher than 95% at 70–80 d after transplantation were cultured for 24 and 48 h in the presence and absence of h-GH and h-IGF-I, followed by determination of expression levels of the eight h-GH-up-regulated genes by real-time qRT-PCR (Fig. 4A). Expression of *IGF-I*, *SOCS2*, *NNMT*, *IGFALS*, and *P4AH1* were significantly increased by h-GH in a dose-dependent manner, but h-IGF-I did not enhance expression of the genes, indicating the direct the action of h-GH on the expression of these genes. The remaining three genes (*KL*, *SLC16A1*, and *SRD5A1*) were not responsive to h-GH or h-IGF-I.

Results for lipogenic genes (*SCD*, *FADS1*, *FADS2*, *FASN*, *DGAT2*, *ADPN*, *AKR1B10*, and *SREBP1c*) are shown in Fig. 4B. Only *FADS1*, *DGAT2*, *SREBP1c*, and *AKR1B10* significantly decreased the expression at 24 or 48 h exposure of 50 ng/ml h-GH. Although insignificant, *SCD*, *FADS2*, and *FASN* were decreased by GH exposure. The expression levels of the eight genes did not significantly change by h-IGF-I.

Discussion

GH regulation of lipogenic genes has been generally studied using rodents (34–37, 41–43), and no suitable animal model whose liver mimics the human liver has been available. Currently, we propose an h-hepatocyte-bearing chimeric mouse as one such model, in which heavy lipid accumulation spontaneously takes place in h-hepatocytes more than 2 months after transplantation but does not when the animals are administered h-GH. Using this model, we demonstrated that h-GH deficiency is a cause of the steatosis and identified 14 and four genes as h-GH-up- and -down-regulated genes at both the hepatocyte and liver tissue level, in which three new lipogenic genes (*FADS1*, *FADS2*, and *AKR1B10*) were included. Regarding h-GH-down-regulated genes, we characterized an additional seven lipogenic genes, although these genes were h-GH down-regulated only at the hepatocyte level or liver tissue level. *FADS1*, *FADS2*, and *AKR1B10* were included in these seven genes and were significantly up-regulated in the chimeric mouse liver compared with human liver, but their expression was down-regulated by h-GH. Thus, it is suggested that these genes participate in the spontaneous steatosis observed in the chimeric mouse liver.

Results of previous studies indicated the presence of species differences in GH responsiveness of lipogenic genes between rats and mice. *SREBP-1c*, a known transcription factor of lipogenic genes, and its target genes, *FASN* and *SCD-1*, appear to be GH up-regulated in rats; hypophysectomy decreases expression of these genes, and the infusion of the rats with GH improved their expression to the original levels (34, 35). By contrast, a study with GH-transgenic technology showed that the same genes were down-regulated in mice (44). Recent microarray analysis supported such species differences; GH treatment suppressed *SCD* gene expression level in hypophysectomized mouse livers (42) but not in hypophysectomized rat livers (41). There are also differences regarding GH responsiveness of lipogenic genes between *in vitro* and *in vivo* studies; the above cited authors showed in a study with primary cultures of rat hepatocytes *SCD1* as GH up-regulated, *FASN* as GH down-regulated, and *SREBP-1c* as GH nonresponsive (34). In the present study, expression of h-*SCD*, but not h-*SREBP-1c*, was reduced by h-GH administration to the chimeric mice, and h-*FABP* expression was not affected by h-GH, which was different from a rat study (34). h-*FADS1*, h-*FADS2*, and h-*AKR1B10* were down-regulated in the present study, but they did not report them as GH-down-regulated genes in rodent studies. In addition, AGHD patients show fatty liver and nonalcoholic steatohepatitis (NASH) (8), and GH treatment improved the symptoms (13, 14). However, studies using hypophysectomized rodents did not report such changes (34, 35, 41, 42).

The serum concentration of GH is low in nonalcoholic fatty liver disease (NAFLD) patients (15), and *NNMT* and

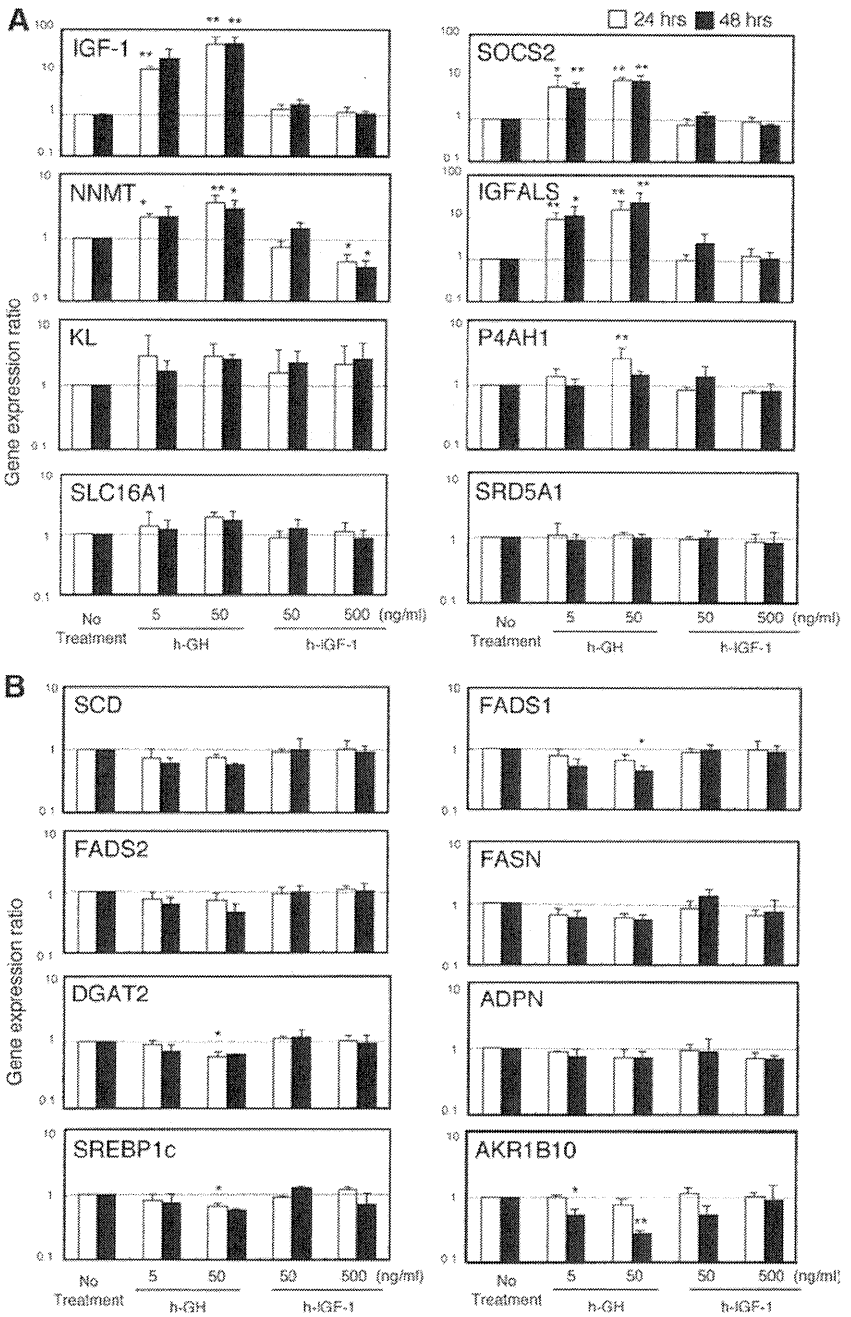

FIG. 4. *In vitro* effects of h-GH and h-IGF-I on the expression level of lipogenesis genes. h-Hepatocytes$_{6Y}$ were cultured with 0, 5, or 50 ng/ml h-GH or 50 or 500 ng/ml h-IGF-I for 24 and 48 h and subjected to RNA isolation to perform real-time qRT-PCR analysis for eight h-GH-up-regulated genes (A) and eight lipogenesis-related genes (B). The expression level of each gene was normalized against that of h-*GAPDH*. The gene expression level of h-hepatocytes treated with h-GH or h-IGF-I was divided by that of untreated h-hepatocytes. *White* and *black bars* represent 24 and 48 h, respectively. Each value represents the mean ± SD. *Asterisks above a bar* show significance between no treatment and each dose of h-GH or h-IGF-I treatment. *, $P < 0.05$; **, $P < 0.01$.

IGFALS are up-regulated in NASH patients (47). Fatty livers of chimeric mice in the present study appreciably reproduce expression profiles of these known NAFLD/NASH-associated genes. We showed that h-GH regulates h-*SCD* and other lipogenesis-related genes, including h-*FADS1*, h-*FADS2*, and h-*AKR1B10* in a h-*SREBP1*-independent manner. Thus, chimeric mice could be particularly useful as an NAFLD/NASH mouse model, with the genes identified in this study serving as therapeutic target genes for NAFLD patients.

Among 14 h-GH-up-regulated genes characterized in this study, eight genes (*IGF-I*, *SOCS2*, *NNMT*, *P4HA1*, *IGFALS*, *MBL2 AMIGO2*, and *SRD5A1*) are known GH-up-regulated genes (23, 41–43, 45), but the remaining six h-GH-up-regulated genes (*ASCL1*, *KL*, *DSCR1L1*, *CREM*, *C5orf13*, and *SLC16A1*) have never been reported as up-regulated genes. Roles of these newly identified GH-up-regulated genes in the human liver could be further investigated using the chimeric mice.

The protein Klotho is known to inhibit insulin/IGF-I signaling, which likely increases resistance to oxidative stress and potentially contributes to its claimed anti-aging properties (46). In the present study, *KL* expression levels were similar in human and chimeric mouse livers, but h-GH markedly induced *KL* gene expression in the latter. The findings of the present study suggested a mutual regulatory mechanism(s) between the two genes: *h-GH* might play a role in the anti-aging process through the *KL* induction.

We were able to propagate h-hepatocytes in chimeric mouse livers, which could solve the problem of a quite limited availability of human hepatocytes for research purposes. In fact, in the present study, we showed the usefulness of chimeric mouse-derived h-hepatocytes for *in vitro* study by testing the effects of h-GH or h-IGF-I on expression levels of eight lipogenic genes that had been up-regulated in chimeric mouse liver *in vivo*. We were able to answer a question of whether h-GH and h-IGF-I in combination directly or indirectly induce such changes in gene expression. Hepatocytes in conventional two-dimensional culture do not generally recapitulate gene expression profiles observed under *in vivo* conditions. In the present study, hepatocytes were three-

dimensionally cultured on Matrigel (spheroid culture), which allowed them to express gene expression closer to *in vivo* conditions as reported previously (48).

We resected h-hepatocyte regions from chimeric livers for microarray analysis and real-time RT-PCR. The gene expression profiles determined using the dissected regions were similar to those determined using the isolated h-hepatocytes$_{\text{chimeric mouse}}$. This finding also indicates the usability of chimeric mouse liver tissues as an alternative RNA source to h-hepatocytes, whose isolation is time consuming and laborious.

In conclusion, the present study shows that chimeric mice could overcome the species difference between experimental animals and humans, and therefore, these mice are useful for investigating the mechanism of the action of GH on h-hepatocytes *in vivo* and role of GH in NAFLD/NASH.

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Address all correspondence and requests for reprints to: Katsutoshi Yoshizato, Ph.D., or Chise Tateno, Ph.D., PhoenixBio. Co. Ltd., 3-4-1 Kagamiyama, Higashihiroshima, Hiroshima 739-0046, Japan. E-mail: katsutoshi.yoshizato@phoenixbio.co.jp or chise.mukaidani@phoenixbio.co.jp.

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Research Article

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ME3738 enhances the effect of interferon and inhibits hepatitis C virus replication both *in vitro* and *in vivo*

Hiromi Abe[1,3], Michio Imamura[1,2], Nobuhiko Hiraga[1,2], Masataka Tsuge[1,2], Fukiko Mitsui[1,2], Tomokazu Kawaoka[1,2], Shoichi Takahashi[1,2], Hidenori Ochi[2,3], Toshiro Maekawa[3], C. Nelson Hayes[1,3], Chise Tateno[2,4], Katsutoshi Yoshizato[2,4], Shoichi Murakami[5], Nobuyuki Yamashita[5], Takashi Matsuhira[5], Kenji Asai[5], Kazuaki Chayama[1,2,3,*]

[1]*Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima-shi 734-8551, Japan;* [2]*Liver Research Project Center, Hiroshima University, Hiroshima, Japan;* [3]*Laboratory for Digestive Diseases, Center for Genomic Medicine, The Institute of Physical and Chemical Research (RIKEN), Hiroshima, Japan;* [4]*PhoenixBio Co., Ltd., Higashihiroshima, Japan;* [5]*Pharmaceutical Research Center, Meiji Seika Kaisha Ltd., Yokohama, Japan*

Background & Aims: ME3738 (22β-methoxyolean-12-ene-3β, 24-diol), a derivative of soyasapogenol B, attenuates liver disease in several animal models of acute and chronic liver injury. ME3738 is thought to inhibit replication of hepatitis C virus (HCV) by enhancing interferon (IFN)-β production, as determined using the HCV full-length binary expression system. We examined the effect of ME3738 combined with IFN-α on HCV replication using the genotype 1b subgenomic replicon system and an *in vivo* mouse HCV model.

Methods: HCV replicon cells (ORN/3-5B/KE cells and Con1 cells) were incubated with ME3738 and/or IFN-α, and then intracellular IFN-stimulated genes (ISGs) and HCV RNA replication were analyzed by reverse-transcription-real time polymerase chain reaction and luciferase reporter assay. HCV-infected human hepatocyte chimeric mice were also treated with ME3738 and/or IFN-α for 4 weeks. Mouse serum HCV RNA titer, HCV core antigen, and ISGs expression in the liver were measured.

Results: ME3738 induced gene expression of oligoadenylate synthetase 1 and inhibited HCV replication in both HCV replicon cells. The drug enhanced the effect of IFN to significantly increase ISG expression levels, inhibit HCV replication in replicon cells, and reduce mouse serum HCV RNA and core antigen levels in mouse livers. The combination treatment was not hepatotoxic as evident histologically and did not reduce human serum albumin in mice.

Conclusions: ME3738 inhibited HCV replication, enhancing the effect of IFN-α to increase ISG expression both *in vitro* and *in vivo*, suggesting that the combination of ME3738 and IFN might be useful therapeutically for patients with chronic hepatitis C.

Keywords: Human hepatocyte chimeric mouse; Interferon-stimulated genes.
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* Corresponding author. Address: Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima-shi 734-8551, Japan. Tel.: +81 82 257 5190; fax: +81 82 255 6220.
E-mail address: chayama@hiroshima-u.ac.jp (K. Chayama).

Abbreviations: HCV, hepatitis C virus; HSA, human serum albumin; IFN, interferon; IL, interleukin; ISG, interferon stimulated gene; MxA, myxovirus resistance protein A; OAS, oligoadenylate synthetase; PKR, double stranded RNA-dependent protein kinase; PCR, polymerase chain reaction; SCID, severe combined immunodeficiency; uPA, urokinase-type plasminogen activator; USP18, ubiquitin specific peptidase 18.

Introduction

The hepatitis C virus (HCV) infects an estimated 170 million people worldwide [1] leading to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [2,3]. To date, the most effective therapy for viral clearance is a 48- or 72-week combination therapy of pegylated interferon (IFN)-α and ribavirin. However, successful eradication of the virus is achieved in only about 50% of treated patients [4–6]. Moreover, therapy induces significant adverse effects, such as fever, fatigue, and anemia [4], resulting in poor tolerability. More effective and less toxic treatment is, therefore, desired.

ME3738 (22β-methoxyolean-12-ene-3β, 24-diol), a derivative of soyasapogenol B [7], attenuates liver disease in several animal models of acute and chronic liver injury induced by concanavalin A, ethanol, lithocholate, and bile duct ligation [8–12]. ME3738 induces interleukin (IL)-6 expression, and serum amyloid A and α1-acid glycoprotein act as downstream targets of the IL-6 signal to protect against concanavalin A-induced liver injury [8–10]. The drug also prevents the progression of hepatic fibrosis in rats with bile duct ligation through suppression of activation and collagen synthesis of hepatic stellate cells [12].

Recently, Hiasa et al. reported that ME3738 inhibited HCV replication by enhancing IFN-β production using the HCV full-length binary expression system that uses full-length genotype 1a HCV complementary DNA plasmid with a T7 promoter sequence and an adenoviral vector expressing T7 polymerase [13]. However, it is not clear if the production of IFN-β and subsequent expression of IFN-stimulated genes (ISGs) was induced by the transcribed HCV genomes through detection by innate

Research Article

immune system receptors, including RIG-I. In addition, it is also not clear whether ME3738 has anti-viral effects on genotype 1b HCV, which is the most common and most IFN-resistant genotype in Japan [14].

Recently, HCV-infected mice have been developed by inoculating HCV-infected human serum into urokinase-type plasminogen activator (uPA)-severe combined immunodeficiency (SCID) mice engrafted with human hepatocytes [15,16]. We and other groups had reported that this mouse model is useful for evaluating anti-HCV drugs such as IFN-α and anti-NS3 protease *in vivo* [17–19].

In the present study, we investigated the effects of ME3738 on HCV replication both *in vitro* and *in vivo* using the genotype 1b HCV replicon and HCV-infected human hepatocyte chimeric mice. The results demonstrate that ME3738 itself had an inhibitory effect on HCV replication, and when combined with IFN, ME3738 enhanced the anti-HCV effect of IFN by up-regulation of ISGs, such as oligoadenylate synthetase (*OAS*) *1*, myxovirus resistance protein A (*MxA*), and *ISG15* in HCV replicon cells. We also showed that the combination therapy increased *OAS1*, RNA-dependent protein kinase (*PKR*) and ubiquitin specific peptidase 18 (*USP18*) expression levels, and reduced virus levels effectively without liver cell damage in human hepatocyte chimeric mice.

Material and methods

Cell culture

Cells supporting replication of the genotype 1b-derived subgenomic HCV replicon, ORN/3-5B/KE cells [20] (kindly provided by N. Kato, Okayama University, Japan) and Con-1 cells [21], were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen Life Technology, Carlsbad, CA) supplemented with 10% fetal bovine serum, non-essential amino acids, glutamine, penicillin, and streptomycin (complete DMEM) in the presence of G418 (300 μg/ml; Geneticin, Invitrogen, Carlsbad, CA). ORN/3-5B/KE and Con1 replicon cells (2×10^4) were seeded onto 12-well plates and incubated for 3 days with or without ME3738 (Meiji Seika Kaisha, Tokyo, Japan) [9], human IFN-α (Dainippon Sumitomo Pharma Co., Tokyo), or the combination of both drugs.

Quantitation of HCV RNA and ISG mRNAs

RNA extraction and quantitation of HCV by real-time polymerase chain reaction (PCR) were performed as described previously [19]. Briefly, RNA was extracted from mice serum, livers, or cellular lysate 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 RNA was performed using the Real-Time PCR system (Applied Biosystems, Foster City, CA). The primers used for amplification were 5′-GAGTGTCGTGCAGCCTCCA-3′ and 5′-CACTCGCAAGCAC CCTATCA-3′. Quantitation of ISGs (*OAS1*, *MxA*, *PKR*, *USP18* and *ISG15*) was performed using real-time PCR Master Mix (TOYOBO) and TaqMan Gene Expression Assay primer and probe sets (PE Applied Biosystems, Foster City, CA). Thermal cycling conditions were as follows: a precycling period of 1 min at 95 °C followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Each ISG expression level was expressed relative to the endogenous RNA levels of the housekeeping reference gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

Luciferase reporter assay

After 72 h of IFN and/or ME3738 treatment, ORN/3-5B/KE cells were harvested with Renilla lysis reagent (Promega, Madison, WI) and subjected to the luciferase assay according to the manufacturer's protocol.

Western blotting

The cells were ruptured with 250 μl lysis buffer [10 mM Tris/HCl pH 7.4, 140 mM NaCl and 0.5% (v/v) NP-40] followed by centrifugation for 2 min at 15,000g. Cell lysates were subjected to Western blotting using antibodies against NS3 (Novocastra Laboratories, UK) and β-actin (Sigma, Tokyo, Japana) as described previously [22].

WST assay

Cell viability was determined by employing tetrazolium salt, WST-8, using the WST-8 Cell Proliferation Assay Kit (Dojindo Laboratories., Kumamoto, Japan), according to the instructions provided by the manufacturer.

Human serum samples

Human serum samples containing high titers of genotype 1b HCV (2.2×10^6 copies/ml) were obtained from a patient with chronic hepatitis after obtaining written informed consent. Aliquots were stored in liquid nitrogen until use.

Animal treatment

All animal protocols in this study were in accordance with the guidelines of the local committee for animal experiments and under approval of the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University. We transplanted human hepatocytes into $uPA^{+/+}/SCID^{+/+}$ mice as described previously [16]. All mice used in this study were transplanted with frozen human hepatocytes obtained from the same donor. Mice were injected intravenously with 50 μl of HCV-positive human serum samples. Six weeks after HCV infection, mice were fed a normal chow containing 0.15% (w/w) ME3738 for 4 weeks, with or without IFN-α. IFN-α-treatment was provided daily by intramuscular injection of diluted IFN solution. Serum samples were collected every week, and human serum albumin (HSA) concentration and HCV RNA were measured. Mouse serum concentrations of HSA, which correlate with the repopulation rates, were measured as described previously [16]. Serum ME3738 concentrations were measured by liquid chromatography/mass spectrometry/mass spectrometry. After the fourth week of treatment, mice were sacrificed, and livers were either fixed with 4% buffered-paraformaldehyde for histological examination or frozen immediately in liquid nitrogen to measure HCV core antigen. To investigate the expression of ISGs in mouse livers, mice were kept for 1 week with or without 0.45% (w/w) ME3738 and then given a single injection of 1500 IU/g IFN-α. Four hours after injection, mice were sacrificed and liver samples were collected.

Quantitation of HCV core antigen in the mouse liver

Livers were homogenized in phosphate-buffered saline with 1% Triton X-100, 0.1% SDS, and 0.5% sodium deoxycholate. The homogenates were centrifuged at 20,000g for 30 min. HCV core antigen levels in the supernatant of liver homogenates were measured using enzyme immunoassay as described previously [23].

Statistical analysis

All data are expressed as mean ± SD. Levels of HCV RNA and ISG mRNAs were compared using the Mann–Whitney *U*-test. A p value less than 0.05 was considered statistically significant. All statistical analyses were performed with SPSS 14.0 software (SPSS, Tokyo, Japan).

Results

Antiviral activity of ME3738 on HCV subgenomic replicon

The effect of ME3738 on HCV replication was analyzed *in vitro* using subgenomic HCV replicon cells possessing the luciferase reporter. ORN/3-5B/KE cells were treated with either IFN-α or ME3738 for 72 h. The luciferase reporter assay demonstrated that the HCV RNA replication level decreased depending on the

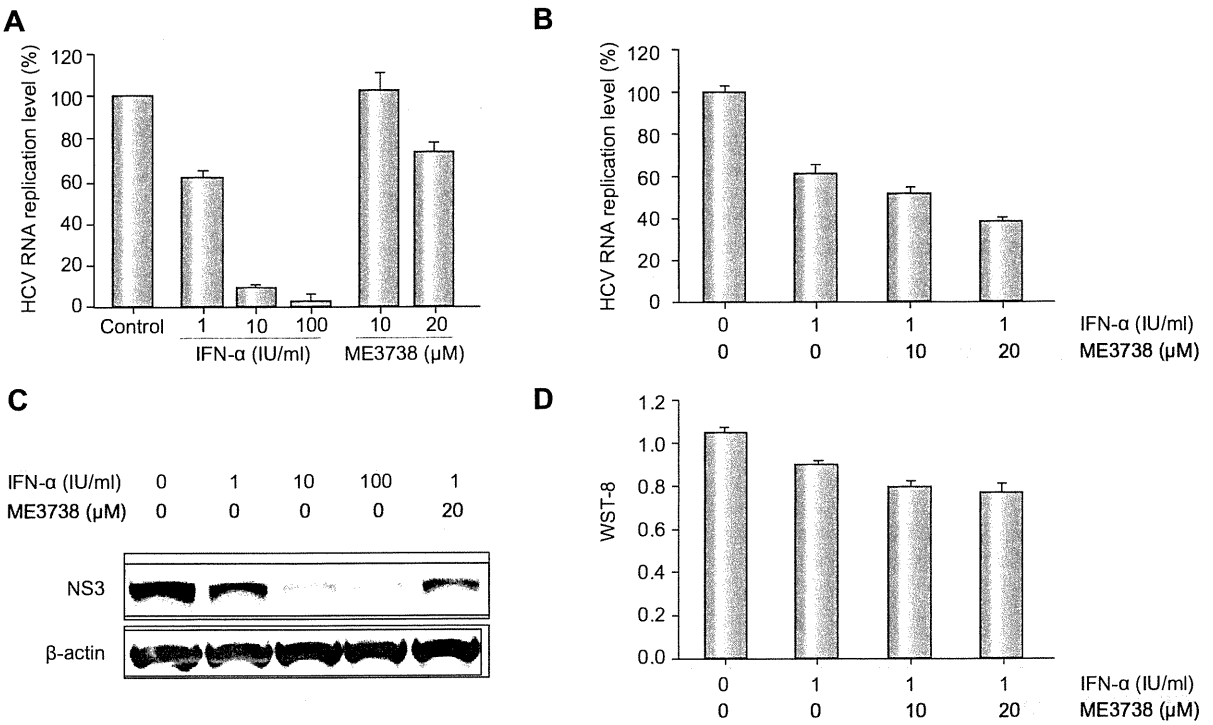

Fig. 1. Effects of ME3738 on HCV replication in the subgenomic HCV replicon, ORN/3-5B/KE cells. ORN/3-5B/KE cells were treated for 72 h with the indicated concentration of interferon (IFN)-α alone, ME3738 alone, or IFN-α plus ME3738. (A and B) Intracellular HCV RNA replication levels were determined as luciferase activity and expressed relative to cellular viability. (C) Cell lysates were analyzed by immunoblotting with antibodies to NS3 and β-actin. (D) Cellular viability was analyzed by WST assay. Data are represented as the mean ± SD of 6 experiments. Control: cells treated with neither ME3738 nor IFN-α.

IFN-treatment dose as reported previously (Fig. 1A) [20]. Treatment with 20 μM of ME3738 also reduced HCV RNA replication. Next, we investigated whether ME3738 enhances the effect of IFN-α. IFN-α (1 IU/ml) plus ME3738 inhibited the HCV RNA replication dose in a dependent manner with ME3738 (Fig. 1B). The level of cellular HCV NS3 protein was reduced depending on IFN-α-treatment and was reduced effectively by IFN-α/ME3738 combination treatment (Fig. 1C). The viability of cells treated with IFN-α/ME3738 combination treatment was lower than that of the control treatment and almost the same as with IFN-α treatment alone (Fig. 1D).

The effect of ME3738 was also tested in a different replicon system, Con-1 cells. ME3738 reduced HCV RNA replication dose dependently in Con-1 cells (Fig. 2A). Similar to ORN/3-5B/KE cells, IFN-α (1 IU/ml) plus ME3738 inhibited HCV RNA replication dose in a dependent manner with ME3738 (Fig. 2A), and the level of cellular HCV NS3 protein was reduced effectively by IFN-α/ME3738 combination treatment (Fig. 2B). The viability of cells treated with IFN-α/ME3738 combination treatment was lower but was not significant with IFN-α treatment alone (Fig. 2C). These results indicate that ME3738 itself has an inhibitory effect on HCV replication and enhances the effect of IFN-α.

Expression of ISGs in ME3738-treated replicon cells

We measured the levels of ISGs in drug-treated ORN/3-5B/KE cells and Con1 cells. IFN-α treatment significantly increased the expression levels of *OAS1*, *MxA*, *PKR*, *USP18* and *ISG15*, which reached maximum levels at 24 h in ORN/3-5B/KE cells (Fig. 3A) and 8 h in Con1 cells (Fig. 3B). ME3738 treatment alone significantly increased the expression of *OAS1* in both cells. IFN-α treatment significantly increased the expression of ISGs; however, IFN-α/ME3738 combination treatment significantly induced the expressions of *OA1S*, *MxA* and *ISG15* to levels higher than IFN-α alone in both cells. These results indicate that ME3738 enhances the effect of IFN-α to increase ISG expression, and this effect may contribute to the inhibition of HCV replication.

Effect of ME3738 on HCV replication in vivo

To further analyze the effects of ME3738, we used genotype 1b HCV-infected human hepatocyte chimeric mice [17,19]. Six weeks after HCV infection, when the mice developed stable viremia (10^6–10^7 copies/ml, data not shown), the animals were treated with ME3738 alone, IFN-α alone, or ME3738/IFN-α for 4 weeks (Fig. 4A). Mouse serum concentrations of ME3738 increased in ME3738- and ME3738 plus IFN-α-treated mice (Table 1). ME3738 alone did not reduce the levels of HCV RNA in mice, while IFN-α-treatment reduced the HCV RNA levels, as reported previously [17]. ME3738 plus IFN-α-treatment significantly reduced HCV to levels lower than that achieved by ME3738 or IFN-α alone. We also measured the HCV core protein level in the livers of treated mice. As shown by replicon experiments, core protein levels were reduced most effectively by the ME3738/IFN-α-combination therapy (Fig. 4B). Since the level of HSA did not decrease in these treatments, it was concluded that

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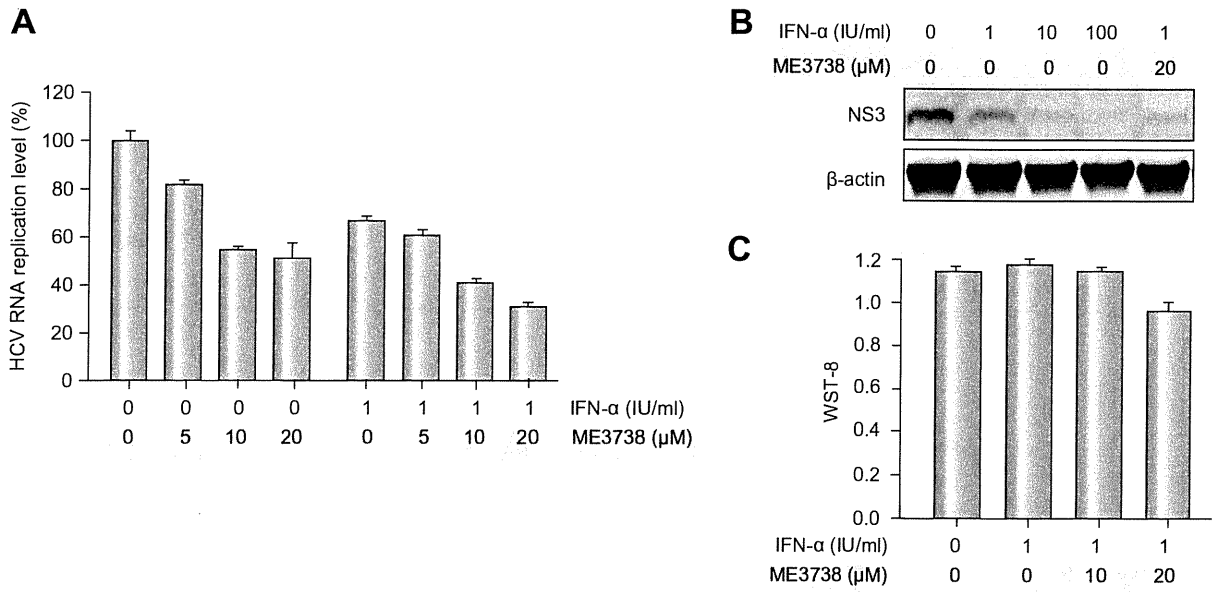

Fig. 2. Effects of ME3738 on HCV replication in the subgenomic HCV replicon, Con1 cells. Con1 cells were treated for 72 h with the indicated concentration of ME3738 alone or IFN-α plus ME3738. (A) Intracellular HCV RNA replication levels were determined via real-time PCR. (B) Cell lysates were analyzed by immunoblotting with antibodies to NS3 and β-actin. (C) Cellular viability was analyzed by WST assay. Data are represented as the mean ± SD of 6 experiments. Control: cells treated with neither ME3738 nor IFN-α.

the reduction of HCV in chimeric mice was not due to toxicity of the drugs (Fig. 4A). This was also supported by histopathological findings, including lack of cytotoxic changes in the livers of all four groups of mice (Fig. 4C). The effect of ME3738 to increase ISG expression was assessed in mouse liver following treatment with a high concentration of ME3738 for 1 week and a single injection of IFN-α. ME3738 alone showed no increase in the expression of ISGs in mouse livers (Fig. 5). IFN-α treatment significantly increased the expression of ISGs; however, IFN-α/ME3738 combination treatment significantly induced the expressions of *OAS1*, *PKR* and *USP18* mRNA levels in mouse livers to levels higher than IFN-α alone. These results indicate that ME3738 inhibits HCV replication, enhancing the effect of IFN-α to increase ISG expression *in vivo*.

Discussion

Although the treatment outcome of chronic HCV infection has improved with the advent of pegylated IFN-α and ribavirin, the eradication rate of HCV is only about 50%. Many patients are unable to receive this therapy because of the harmful side effects or the financial costs. Development of effective, safe and inexpensive therapies should be encouraged.

ME3738 is reported to attenuate various liver pathologies in animals [8–12]. Furthermore, Hiasa et al. reported recently that ME3738 induces IFN-β mRNA expression and inhibits the replication of HCV [13]. We thus attempted in this study to evaluate the effect of ME3738, especially in combination with IFN-α, on HCV.

The results of the present study show that ME3738 induced the gene expression of *OAS* (Fig. 2) and inhibited HCV replication (Fig. 1A). Hiasa et al. reported that ME3738 enhanced the expression of *IFN-β* mRNA and that the enhanced production of IFN-β resulted in the increased expression of ISGs [13]. They showed also that the effect of ME3738 on HCV was abolished following the inhibition of IFN-β expression with siRNA or antibody. Our results are consistent with their findings. The extent of the increase in ISG expression was smaller in Hiasa et al. [13] than in our results. This is probably because they used the T7-genotype 1a-cDNA transient transfection-infection system to produce HCV in HepG2 or Huh7 cells [13,24,25] and assessed the effect of ME3738 by utilizing naturally produced IFN-β. The amount of IFN is likely to be very small in their system compared to that used in our study. We also tried to detect *IFN-β* mRNA in our replicon system but were unable to detect it in our replicon cells (Huh7 based ORN/3-5B/KE cells and Con1 cells). This is probably due to a defect of the innate immune system in producing IFN-β in those cells. This is consistent with their finding that ME3738 had an inferior effect in Huh7 cells than in HepG2 cells to produce ISG products [13].

As we showed in this study, ME3738 enhances the effect of IFN against HCV replication both *in vitro* (Figs. 1B and 2A) and *in vivo* (Figs. 4A and 4B). ME3738 enhanced the effect of IFN-α by increasing the expression levels of ISGs both *in vitro* (Fig. 3) and *in vivo* (Fig. 5). How ME3738 enhances the transcription of ISGs is unknown at this stage. ME3738 was reported initially to protect liver cells against injury through induction of IL-6 [8,9]. IL-6 is reported to provide protection to certain cells [26–28] by preventing apoptosis. In the present study, we tried to detect IL-6 protein in the serum and mRNA in the liver of ME3738-treated mice. However, the levels of both were too low to measure. Further studies should be conducted to elucidate the mechanism by which ME3738 enhances immunity against viral infections.

Our results showed that ME3738 did not reduce cell viability. We also showed that the drug is not hepatotoxic, as inferred by HSA level and liver histology. Since ME3738 is reported to

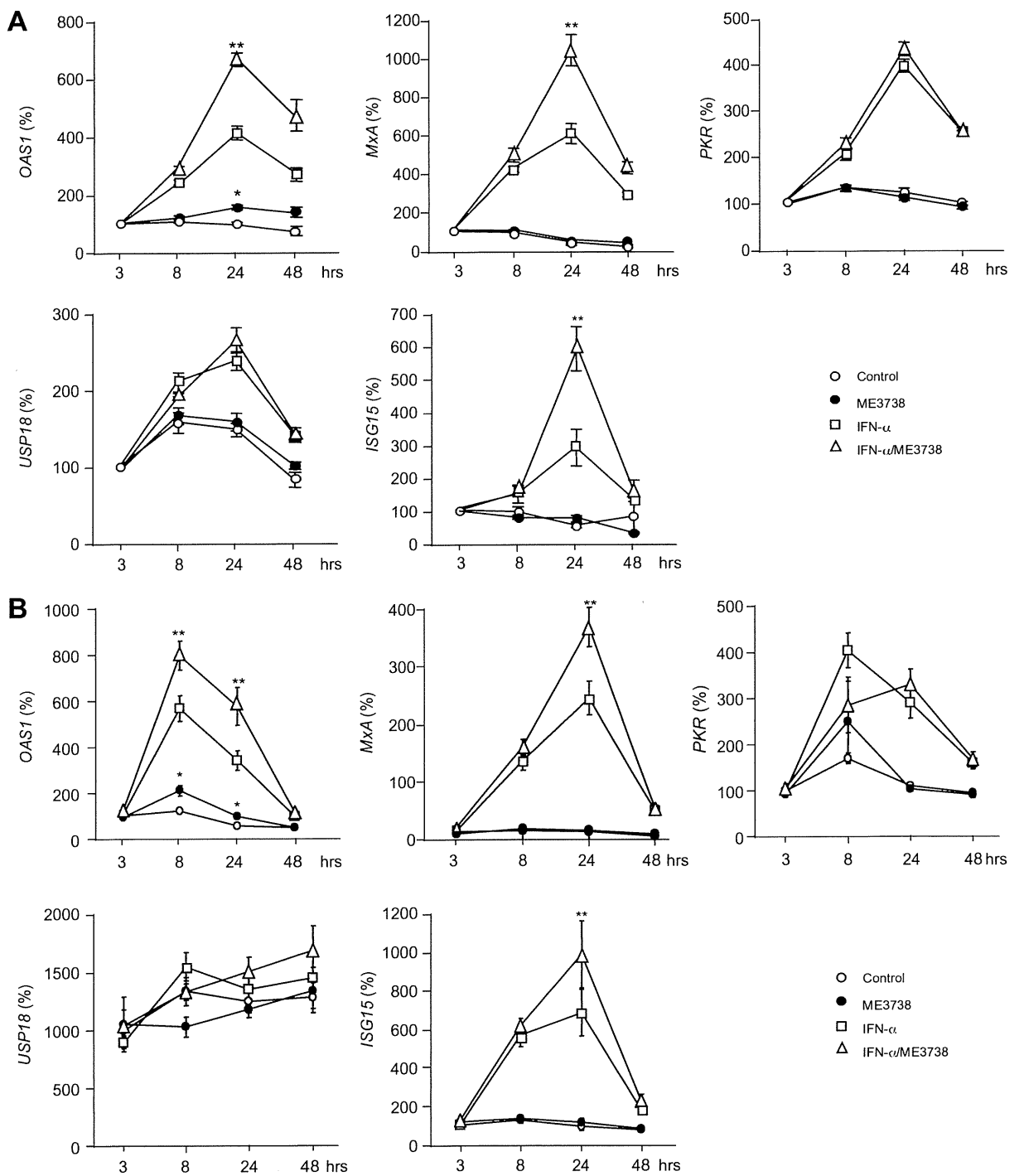

Fig. 3. Effects of ME3738 on the expression of interferon-stimulated genes. ORN/C-5B/KE cells (A) and Con1 cells (B) were treated with 20 μM of ME3738 and/or 1 IU/ml of interferon (IFN)-α for 48 h. Intracellular gene expression levels of oligoadenylate synthetase (*OAS*), myxovirus resistance protein A (*MxA*), double stranded RNA-activated protein kinase (*PKR*), *USP-18* and interferon-stimulated gene (*ISG*) *15* were measured at the indicated times. RNA levels were expressed relative to *GAPDH* mRNA. Data are shown as the mean ± SD of 6 experiments. Control: cells treated with neither ME3738 nor IFN-α. (*p <0.05 compared with Control, **p <0.05 compared with IFN-α treatment).

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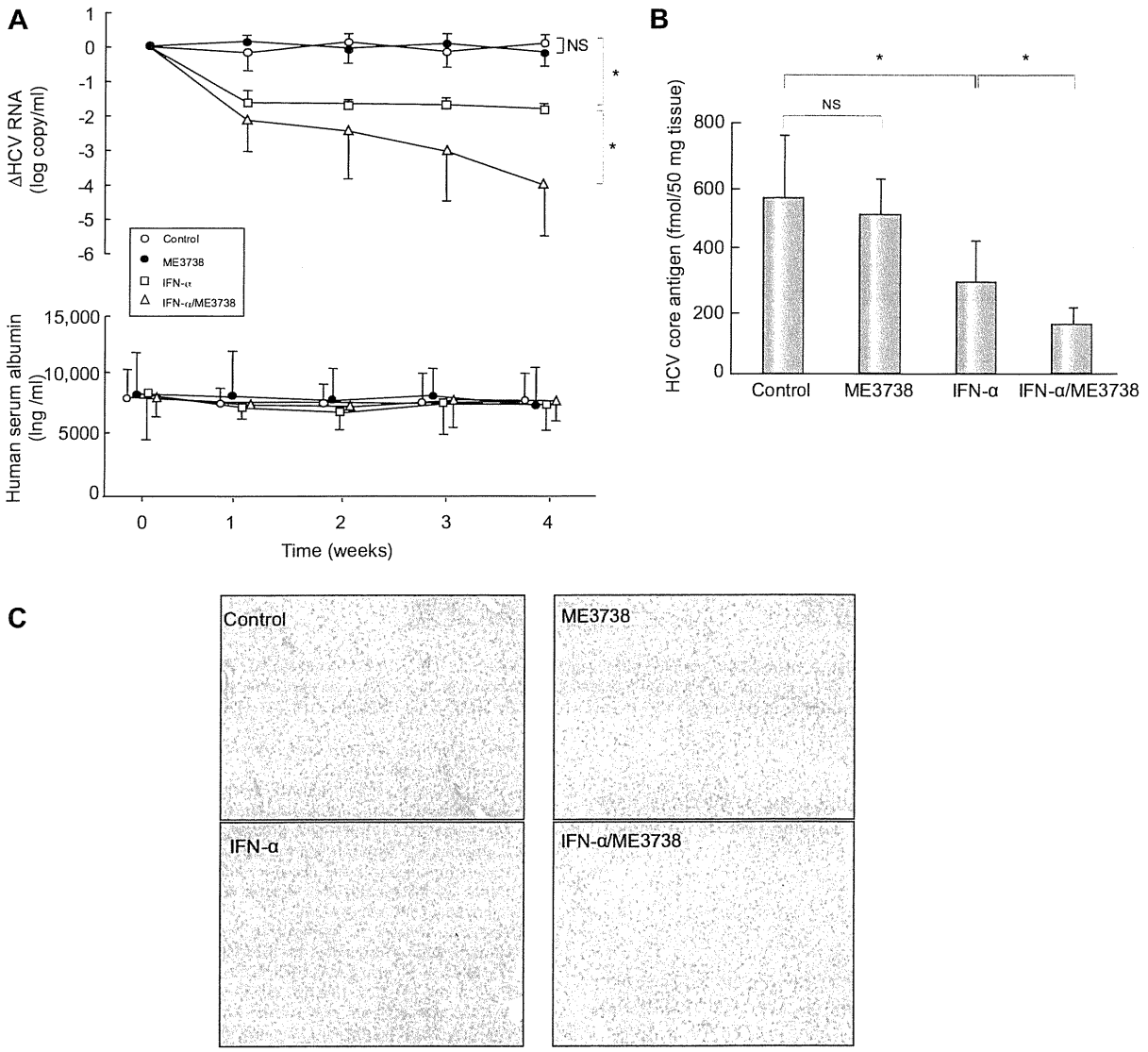

Fig. 4. ME3738 enhances the effect of IFN in mice with HCV infection. Mice were injected intravenously with 50 μl of HCV-positive human serum samples. Six weeks after HCV infection, mice were treated with ME3738 and/or interferon (IFN)-α for 4 weeks. (A) Mouse serum samples were obtained every week, and HCV RNA titer (upper panel) and human serum albumin concentration (lower panel) were analyzed. (B) HCV core antigen was measured in the mouse livers after 4 weeks of treatment. Data are mean ± SD of 6 mice. (*p <0.05; **p <0.01; NS, not significant). (C) Liver samples obtained from mice were stained with hematoxylin-eosin (Original magnification, 100×). Note the lack of specific changes in the mice of each group. Control: HCV-infected mice treated with neither ME3738 nor IFN-α.

Table 1. Concentrations of ME3738 in mouse serum samples.

	Control	ME3738	IFN-α	ME3738/ IFN-α
ME3738 (μM)	<0.01	4.02 ± 0.90	<0.01	2.44 ± 0.21

Concentrations of ME3738 in serum samples obtained from mice after 4 weeks of treatment were measured by liquid chromatography/mass spectrometry/mass spectrometry. Data are shown as mean ± SD of three mice. Control: HCV-infected mice treated with neither ME3738 nor IFN-α.

attenuate liver disease in several animal models of acute and chronic liver injury [8–12], the drug could be suitable for treatment of patients with chronic hepatitis C. In the current regimen of PEG-IFN and ribavirin combination therapy, IFN reduces the replication rate of the virus by inducing expression of ISGs in liver cells. Ribavirin enhances the effect of IFN synergistically through an unknown mechanism. ME3738 also enhances the effect of IFN similarly to ribavirin and may protect liver cells from apoptosis. Combination therapy using these three drugs might yield excellent anti-viral and anti-inflammatory effects. Alternatively, ME3738 could be used instead of ribavirin if the drug shows a superior effect in combination with IFN. Further animal and human studies should be conducted to develop an effective regimen for the treatment of patients with chronic hepatitis C.

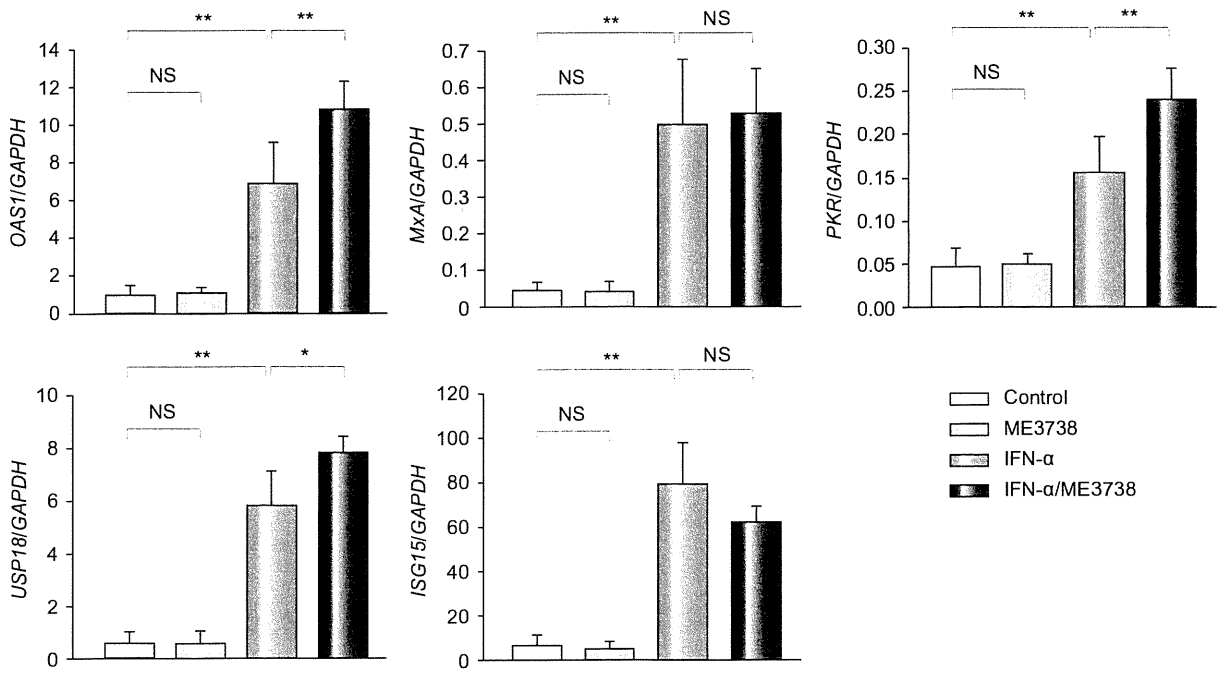

Fig. 5. Interferon-stimulated gene expression in mouse liver samples. Mice were treated with or without 0.45% (w/w) ME3738 for 1 week and then given a single injection of 1500 IU/g IFN-α. Four hours after IFN-α injection, interferon stimulated gene expression in mouse livers was measured. RNA levels are expressed relative to *GAPDH* mRNA. Data are presented as mean ± SD of six mice. Control: Mice treated with neither ME3738 nor IFN-α. (*p <0.05; **p <0.01; NS, not significant).

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

Eiji Ohara[1,2], Nobuhiko Hiraga[1,2], Michio Imamura[1,2], Eiji Iwao[3], Naohiro Kamiya[3], Ichimaro Yamada[3], Tomohiko Kono[1,2], Mayu Onishi[1,2], Daizaburo Hirata[1,2], Fukiko Mitsui[1,2], Tomokazu Kawaoka[1,2], Masataka Tsuge[1,2], Shoichi Takahashi[1,2], Hiromi Abe[1,2], C. Nelson Hayes[2,4], Hidenori Ochi[2,4], Chise Tateno[2,5], Katsutoshi Yoshizato[2,5], Shinji Tanaka[1], Kazuaki Chayama[1,2,4,*]

[1]*Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan;* [2]*Liver Research Project Center, Hiroshima University, Hiroshima, Japan;* [3]*Pharmacology Research Laboratories I, Mitsubishi Tanabe Pharma Corporation, Yokohama, Japan;* [4]*Laboratory for Digestive Diseases, Center for Genomic Medicine, RIKEN, Hiroshima, Japan;* [5]*PhoenixBio Co., Ltd., Higashihiroshima, Japan*

See Editorial, pages 848–850

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.

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

Keywords: NS3-4A protease inhibitor; NS5B RNA polymerase inhibitor; Human hepatocyte chimeric mouse; Interferon.
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* Corresponding author at: Department of Medical and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate school of Biomedical Science, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. Tel.: +81 82 257 5190; fax: +81 82 255 6220.
E-mail address: chayama@hiroshima-u.ac.jp (K. Chayama).

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.

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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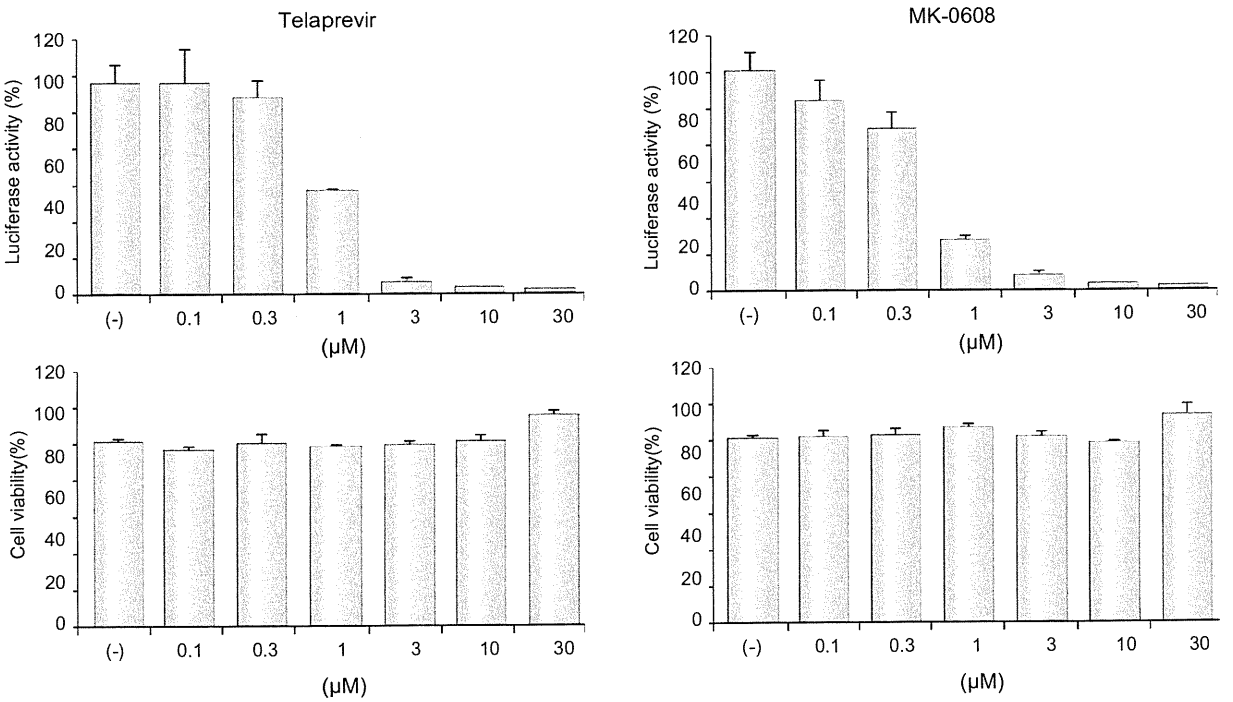

Fig. 1. *In vitro* analysis of susceptibility of HCV replicon cells to anti-HCV drugs. Huh7/Rep-Neo cells were treated for 72 h with the indicated concentrations of either telaprevir or MK-0608. Intracellular HCV RNA replication levels were determined as luciferase activities (upper panel), and expressed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

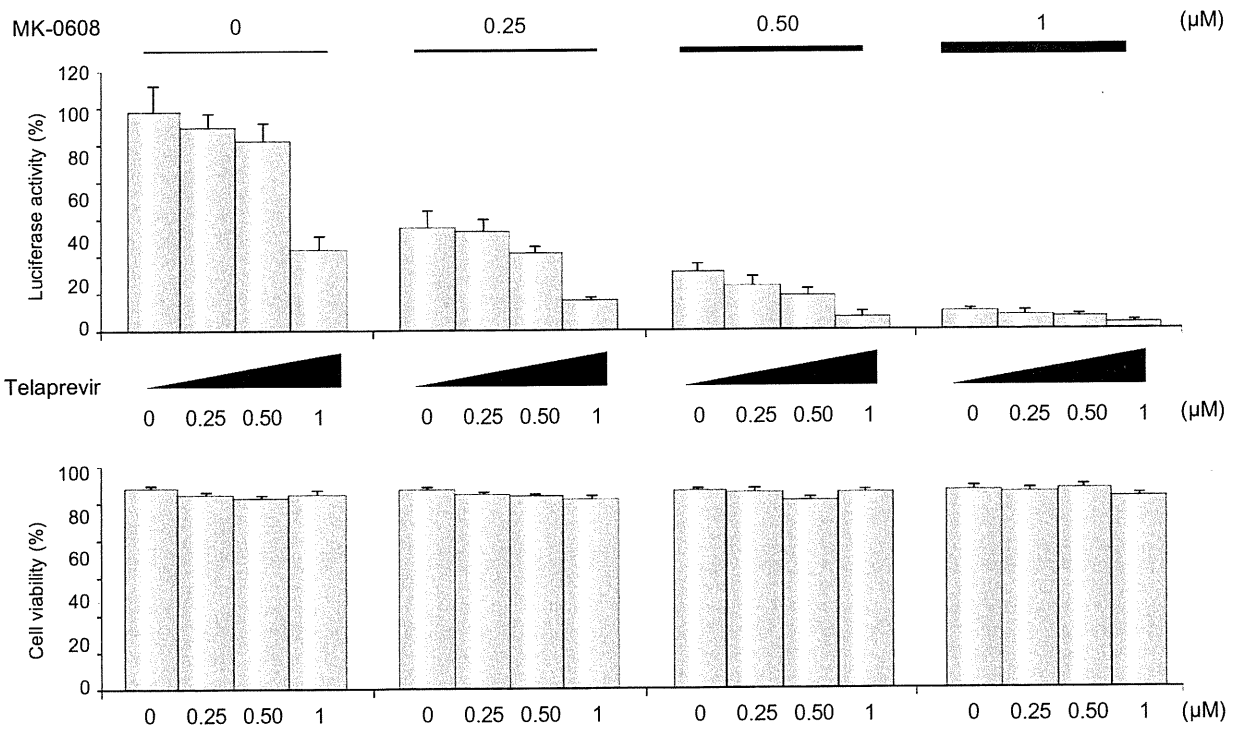

Fig. 2. *In vitro* analysis of susceptibility of HCV replicon cells to combination treatment with anti-HCV drugs. Huh7/Rep-Neo cells were treated for 72 h with the indicated concentration of MK-0608 plus telaprevir. Intracellular HCV RNA replication levels were determined as luciferase activities (upper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