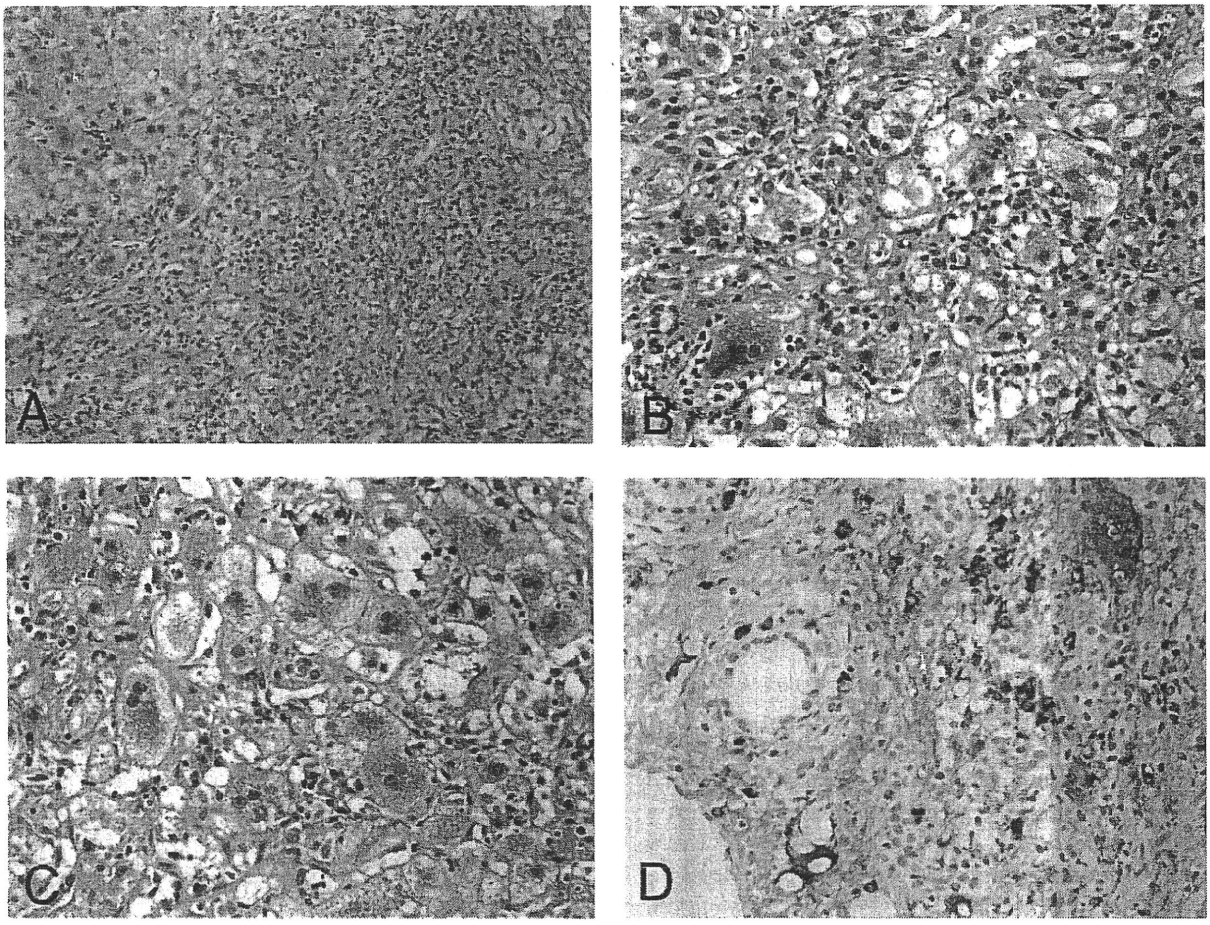
Another Cause of Autoimmune Hepatitis



A 42-year-old man was admitted to our hospital because of elevated liver enzymes (aspartate aminotransferase, 642 IU/L [normal range: 12-37]; alanine aminotransferase, 788 IU/L [normal range: 7-45]; alkaline phosphatase, 605 IU/L [normal range: 124-367]; γ-glutamyl transpeptidase, 180 IU/L [normal range: 6-30]; and total bilirubin, 8.6 mg/dL [normal range: 0.3-1.2]). His serum immunoglobulin G (IgG) concentration was 5622 mg/dL (normal range: 870-1700), and anti-nuclear antibody titer (1:20480), anti-double-stranded DNA (>400 IU/mL), and smooth muscle antibody titer (1:40) were all abnormal. Infection with hepatitis A, B, and C; cytomegalovirus; and Epstein-Barr virus were excluded, and no drug use was noted. Ultrasonography, abdominal computed tomography, and magnetic resonance imaging showed no abnormalities of the extrahepatic

Abbreviations: AIH, autoimmune hepatitis; HE, hematoxylin and eosin; IgG, immunoglobulin G.

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bile ducts or pancreas. The first liver biopsy showed changes associated with typical autoimmune hepatitis (AIH); liver parenchyma was collapsed with broad fibrous septa containing entrapped hepatocytes, and lymphoplasmacytic infiltration with interface activity was seen (Fig. 1A; hematoxylin and eosin [H&E] staining, magnification ×200). Hepatocytes showed rosetting in numerous places (Fig. 1B; H&E staining, magnification ×400). Lobular inflammation was evident with giant cell change of hepatocytes (Fig. 1C; H&E staining, magnification ×400), but no biliary epithelial changes were found. The patient fulfilled the criteria for definite AIH by the International Autoimmune Hepatitis Group and was administered corticosteroids at 60 mg/day, which led to improvement of laboratory findings. Prior to treatment, however, the patient's serum IgG4 concentration was 642 mg/dL (normal: ≤ 135) in a stored serum sample, and immunostaining of liver tissue showed abundant plasma cells with strong immunohistochemical reactivity to IgG4 in a portal tract (Fig. 1D; IgG4 immunostaining, magnification ×400). A second liver biopsy performed 7 months afterward showed remaining portal sclerosis, but lobular distortion and portal inflammation were ameliorated, and serum alanine aminotransferase and IgG4 concentrations were normalized. IgG4-positive plasma cells were scarce in portal tracts (data not shown).

In an earlier report, a strong and unexpected association was seen between serum IgG4 concentration and IgG4-bearing plasma cell infiltration in the liver of a case with type 1 AIH, raising the possibility of a new disease entity termed IgG4-associated AIH.[1] Raised serum IgG4 concentration and IgG4-bearing plasma cell infiltration have a high sensitivity and specificity for the diagnosis of IgG4-related diseases.[2-4] Similar to the present case, histological findings in the liver of patients with IgG4-associated AIH showed bridging fibrosis, portal inflammation with abundant plasma cell infiltration, interface hepatitis, and lobular hepatitis. More interestingly, giant cell change and rosette formation were obvious as well. These two cases imply that IgG4-related inflammatory processes can occur in the hepatic parenchyma similarly to those in the pancreatobiliary system, and such cases may resemble AIH both clinically and pathologically. On the contrary, Chung et al. described IgG4-associated AIH as patients with AIH who had IgG4-positive plasma cells in the liver.[5] Because no cases showed high serum IgG4 in their cohort, we believe they are different from our two representative patients and thus should not be classified as an IgG4-related disease. Koyabu et al. recently reported that an IgG4/IgG1-bearing plasma cell ratio of >1 in the liver is specific for IgG4-related diseases.[6] In our patient, the IgG4/IgG1 ratio was >1 (data not shown) and consistent with their findings, which provides further evidence of our case as an IgG4-related disorder. Because IgG4-associated AIH is clearly an IgG4 hepatopathy, this disease should be differentiated from classical AIH. Detection of IgG4 and assessment of liver histology using IgG4 immunostaining may be useful for distinguishing IgG4-related diseases from classical AIH.

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Research Article

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Association analysis of *cytotoxic T-lymphocyte antigen 4* gene polymorphisms with primary biliary cirrhosis in Japanese patients

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Background & Aims: Primary biliary cirrhosis (PBC) is an organ-specific autoimmune disease of still unidentified genetic etiology that is characterized by chronic inflammation of the liver. Since *cytotoxic T-lymphocyte antigen 4* (*CTLA4*) polymorphisms have recently been linked with PBC susceptibility in studies on Caucasians, we investigated the genetic association between *CTLA4* polymorphisms and PBC in a Japanese population.

Methods: Five single nucleotide polymorphisms (SNPs) in the *CTLA4* gene (rs733618, rs5742909, rs231775, rs3087243, and rs231725) were genotyped in 308 patients with PBC and 268 healthy controls using a TaqMan assay.

Results: One *CTLA4* gene SNP (rs231725) was significantly associated with susceptibility to anti-mitochondrial antibody (AMA)-positive PBC, but clinical significance disappeared after correction for multiple testing. Moreover, *CTLA4* gene SNPs did not influence AMA development or disease progression to orthotopic liver transplantation in our Japanese cohort. In haplotype analyses, one haplotype [haplotype 1 (CGGA)] at rs5742909, rs231775, rs3087243, and rs231725, was significantly associated with susceptibility to both AMA-positive PBC and overall PBC.

Conclusions: This study showed that *CTLA4* gene polymorphisms had a modest, but significant association with susceptibility to PBC in the Japanese population. The connection between genetic variants and the function of the *CTLA4* gene remains to be addressed in future investigations.

Keywords: Primary biliary cirrhosis; Single nucleotide polymorphisms; Cytotoxic T-lymphocyte antigen 4; Genetic susceptibility.

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Abbreviations: PBC, primary biliary cirrhosis; AMA, anti-mitochondrial antibody; CTLA4, cytotoxic T-lymphocyte antigen 4; OLT, orthotopic liver transplantation; SNPs, single nucleotide polymorphisms; UTR, untranslated region; LD, linkage disequilibrium; HWE, Hardy–Weinberg equilibrium; *pc*, corrected *p*; OR, odds ratio; CI, confidence interval; sCTLA4, soluble isoform of CTLA4.

Introduction

Primary biliary cirrhosis (PBC) is a liver-specific autoimmune disease characterized by female preponderance and the destruction of intrahepatic bile ducts that often results in cirrhosis and hepatic failure [1]. The etiology of PBC has yet to be conclusively elucidated, although genetic factors are considered to play a prominent role in family and population studies [2–5]. Prior reports have shown the HLA-*DRB1*08* allele to be a weak and regional determinant of PBC susceptibility [6–8]. However, HLA alone does not explain the entire genetic predisposition to PBC, mainly because at least 80–90% of patients with the disease do not carry the most common HLA susceptibility alleles. In this regard, other non-HLA genes are thus being considered to contribute to disease development [9,10].

PBC displays immunologically characteristic features like biliary lymphocytic infiltrates, anti-mitochondrial antibodies (AMA) against the inner lipoyl domain of the E2 subunits of the pyruvate dehydrogenase complex, and elevated serum levels of IFN-γ and TNF-α. The serologic hallmark of PBC is the presence of AMA [11,12], which are found in 95% of patients with PBC [13] and have a specificity of 98% for the disease [12]. Auto-reactive $CD4^+$ and $CD8^+$ T cells are also found in high concentrations in the portal triads of patients with PBC, often surrounding and infiltrating necrotic bile ducts [14–16]. A recent study suggested that a reduction in the number of $CD4^+CD25^+$ regulatory T cells in livers affected with PBC contributed to disease progression [17]. Accumulating data such as these, support a direct role of T-lymphocytes in the pathogenesis of PBC.

The cytotoxic T-lymphocyte antigen 4 (CTLA4) is an inhibitory receptor expressed on the cell surface of activated memory T cells and $CD4^+CD25^+$ regulatory T cells that acts largely as a negative regulator of T-cell responses. Since the potential inhibitory functions of CTLA4 [18] may also trigger a breakdown of immunological self-tolerance, polymorphisms affecting these processes could have significant effects on susceptibility to autoimmunity.

The *CTLA4* gene is a primary candidate for genetic susceptibility to autoimmune diseases, including type 1 diabetes, auto-

immune hepatitis [19,20], and autoimmune pancreatitis [21]. In particular, two single nucleotide polymorphisms (SNPs), rs231775 (49AG) and rs3087243 (CT60), have been widely studied in PBC [22–24]. Although early studies found an association between SNP 49G coding and PBC [22–24], ensuing reports showed negative relationships with susceptibility [25–30] or a positive association with liver damage [31]. A recent investigation reported that rs231725 in the 3′ flanking region of *CTLA4* is associated with AMA-positive PBC in Caucasians [27]. In addition to *CTLA4* polymorphisms, HLA class II, IL12A, IL12RB, and several other candidate SNPs were disclosed as predisposition genes for PBC by a high-density genome-wide association study [9]. Since these SNPs have not been extensively examined in a large Japanese population, the present study sought to evaluate the involvement of *CTLA4* SNPs and haplotype SNPs in susceptibility to PBC and disease progression in Japanese patients.

Patients and methods

Subjects

We analyzed a total of 576 subjects (308 PBC patients and 268 healthy controls) collected from two different regions of Japan (Table 1). Cohort 1 consisted of 198 patients clinically diagnosed with PBC (173 women, median age 58 years old) and 170 healthy subjects who were seen at Shinshu University Hospital, Matsumoto, Japan. Cohort 2 consisted of 110 patients clinically diagnosed with PBC (92 women, median age 61 years old) and 98 healthy subjects from the National Hospital Organization Nagasaki Medical Center, Omura, Japan. The racial background of all subjects was Japanese. Control subjects were volunteers from hospital staff who had indicated the absence of any major illnesses in a standard questionnaire. The diagnosis of PBC was based on criteria from the American Association for the Study of Liver Diseases [32]. Serum AMA, specific for the pyruvate dehydrogenase complex-E2 component, was measured by the enzyme-linked immunosorbent assay as reported previously [33]. An index of greater than seven was considered a positive result. All patients were negative for hepatitis B surface antigen, antibody to hepatitis C virus, and antibody to human immunodeficiency virus. To evaluate associations between SNPs and disease progression, patients were classified into two stages based on their most recent follow-up [34]: early stage patients were histologically in Scheuer stage I or II [35,36] or of unknown histological stage without liver cirrhosis, and late stage patients were histologically in Scheuer stage III or IV or clinically diagnosed with liver cirrhosis or hepatic failure. All participants provided informed written consent for this study, which had been approved by the institutional ethics committee.

CTLA4 SNP genotyping

Genomic DNA from patients and controls was isolated by phenolic extraction of sodium dodecyl sulfate-lysed and proteinase K-treated cells, as described previously [37,38], and adjusted to 10–15 ng/μl.

The five *CTLA4* gene SNPs examined in this study (rs733618, rs5742909, rs231775, rs3087243, and rs231725) were genotyped using the 5′ nuclease (TaqMan) assay using primer, probes, and reaction conditions as recommended by the manufacturer (Applied Biosystems, Tokyo, Japan). These SNPs were selected based on previous reports [21–23,26,27], and were all located in the *CTLA4* gene; SNPs rs733618 and rs5742909 were in the promoter region, SNP rs231775 in exon 1, and SNPs rs3087243 and rs231725 in the 3′ untranslated region (UTR). Polymerase chain reaction was performed with a TaqMan Assay for Real-Time PCR (7500 Real-Time PCR System; Applied Biosystems) following the manufacturer's instructions.

Haplotype–genotype estimation

The R package "haploview" [39] was used to evaluate the haplotype structure of the five examined *CTLA4* SNPs. Pairwise linkage disequilibrium (LD) patterns and haplotype frequency analysis for all SNPs in patients and controls were assessed by the block definition by Gabriel et al. [40].

Table 1. Demographic and clinical data of patients with PBC at study onset.

Characteristics	Cohort 1 Shinshu $n = 198$	Cohort 2 Nagasaki $n = 110$	Combined $n = 308$
Age, years[a]	58 (30–83)	61 (34–85)	58 (30–88)
Female/Male	173/25	92/18	265/43
Disease progression			
Early stage, *n*/Late stage, *n*	149/49	74/36	223/85
Orthotopic liver transplantation, *n* (%)	15 (7.6)	2 (1.8)	17 (5.5)
AMA positive, *n* (%)	171 (86.4)	102 (92.8)	273 (88.6)

PBC, primary biliary cirrhosis; AMA, anti-mitochondrial antibody specific for the pyruvate dehydrogenase complex-E2 component.
[a] Median (range).

Statistical analysis

The Hardy–Weinberg equilibrium (HWE) test was done for each SNP between control and patient groups. The significance of allele distribution between PBC patients and healthy controls was assessed using the χ^2-test with the use 2 × 2 or 2 × 3 comparisons. Fisher's exact probability test was used for groups with fewer than 5 samples. A p value of less than 0.05 was considered statistically significant; p values were corrected using Bonferroni's correction by multiplying by the number of different alleles observed in each locus (pc).

Results

In total, five SNPs located in the *CTLA4* gene were genotyped in 198 patients with PBC and 170 healthy controls in cohort 1 and 110 patients with PBC and 98 healthy controls in cohort 2 (Table 2). Hardy–Weinberg equilibrium (HWE) was observed for all 5 of the examined SNPs in both control groups, and the minor allele frequencies of all SNPs were greater than 5%. In cohort 1, one SNP (rs733618) differed significantly from HWE ($p = 0.03$) (Table 2), and the frequency of the minor A allele at rs231775 was significantly decreased (33.9% vs. 41.5%, odds ratio (OR) 0.72, 95% confidence interval (95% CI) 0.53–0.99, $p = 0.042$, $pc = 0.209$) in 171 AMA-positive PBC patients compared with controls. Positivity for the major G allele (A/G + G/G) at rs231775 was significantly higher in patients with AMA-positive PBC than in healthy subjects (88.3% vs. 79.1%, OR 1.96, 95% CI 1.08–3.53, $p = 0.026$, $pc = 0.128$). Additionally, the allele frequency (61.7% vs. 53.2%, OR 1.41, 95% CI 1.04–1.92, $p = 0.025$, $pc = 0.127$) and allele carrier frequency (86.0% vs. 75.9%, OR 1.96, 95% CI 1.12–3.41, $p = 0.018$, $pc = 0.089$) of the major A allele at rs231725 were significantly increased in AMA-positive PBC patients compared with healthy controls. However, these statistical significances disappeared after correction for multiple testing. No significant differences were observed among the 5 SNPs in cohort 2. The allele frequency (60.3% vs. 53.4%, OR 1.33, 95% CI 1.04–1.69, $p = 0.022$) of the major A allele at rs231725 was significantly increased in combined analysis (cohorts 1 and 2) of 273 AMA-positive PBC patients compared with 268 healthy controls (Table 3), but statistical significance was lost after correction for multiple testing ($pc = 0.110$) (Table 3).

Pairwise LD mapping confirmed that all alleles were in strong LD with an index of >0.8. A strong LD was detected in the same block for PBC patients and controls. We next evaluated haplotype association among AMA-positive PBC patients and healthy subjects in a combined analysis. To estimate haplotype frequencies and analyze haplotype association with PBC, we selected tag SNPs

Table 2. Allele frequencies of SNPs in the *CTLA4* gene in PBC patients and controls.

SNP No.	dbSNP	Allele major/minor	Position (bp)	Gene location	Cohort 1 (Shinshu)				Cohort 2 (Nagasaki)			
					Patients (n = 198)		Controls (n = 170)		Patients (n = 110)		Controls (n = 98)	
					MAF (%)	HWE p value	MAF (%)	HWE p value	MAF (%)	HWE p value	MAF (%)	HWE p value
1	rs733618	T/C	204439189	Promoter	44.4	0.030	39.1	0.071	39.5	0.570	43.4	0.366
2	rs5742909	C/T	204440592	Promoter	9.1	0.347	11.2	0.295	13.2	0.828	13.8	0.514
3	rs231775	G/A	204440959	Exon 1	35.4	0.784	41.5	0.089	39.5	0.334	41.8	0.827
4	rs3087243	G/A	204447164	3′ UTR	26.3	0.994	30.3	0.709	26.4	0.125	31.1	0.316
5	rs231725	A/G	204448920	3′ UTR	39.9	1.000	46.8	0.288	41.8	0.586	46.4	1.000

MAF, minor allele frequency; HWE, Hardy–Weinberg equilibrium; UTR, untranslated region.

Table 3. Allele frequencies of 5 SNPs in 273 AMA$^+$ patients with PBC and 268 healthy subjects.

SNP No.	Allele	Patients*	Controls*	p	pc	OR	95% CI
1	C	43.2	40.7	0.395	1.975	1.11	0.87–1.41
	T	56.8	59.3				
2	C	89.6	87.9	0.380	1.900	1.18	0.81–1.73
	T	10.4	12.1				
3	G	63.9	58.4	0.062	0.310	1.26	0.99–1.61
	A	36.1	41.6				
4	G	74.4	69.4	0.070	0.350	1.28	0.98–1.67
	A	25.6	30.6				
5	A	60.3	53.4	0.022	0.110	1.33	1.04–1.69
	G	39.7	56.6				

AMA, anti-mitochondrial antibodies; PBC, primary biliary cirrhosis; OR, odds ratio; pc, corrected p value; 95% CI, 95% confidence interval; *, frequency (%).
p value was calculated by a χ^2-test 2 × 2 contingency table (df = 1).

Table 4. *CTLA4* haplotypes in 273 AMA$^+$ patients with PBC and 268 healthy subjects.

Haplotype	SNP No.				Patients* (n = 546)	Controls* (n = 536)	p	OR	95% CI
	2	3	4	5					
1	C	G	G	A	59.7	51.9	0.0095	1.37	1.08–1.75
2	C	A	A	G	25.5	29.4	0.1464	0.82	0.62–1.07
3	T	A	G	G	10.3	11.8	0.4186	0.85	0.58–1.25
4	C	G	G	G	3.8	5.4	0.2153	0.70	0.39–1.23

PBC, primary biliary cirrhosis; OR, odds ratio; 95% CI, 95% confidence interval; *, proportion of indicated haplotype (%).
Values for n indicate two times the number of individuals since each person carries two haplotypes.
p value was calculated by a χ^2-test 2 × 2 contingency table (df = 1).

using the Tagger algorithm from the Haploview program. Four tag SNPs (SNPs 2–5: rs5742909, rs231775, rs3087243, and rs231725) were selected to capture most of the allelic diversity in the two cohorts. The four estimated haplotypes showed a frequency of >5% in 11 haplotypes created by expectation–maximization algorithms (Table 4). Haplotype 1 (CGGA) was significantly associated with AMA-positive PBC susceptibility (59.7% vs. 51.9%, OR 1.37, 95% CI 1.08–1.75, p = 0.0095). No other haplotypes were associated with either susceptibility or resistance to PBC.

Evaluation of the 5 *CTLA4* SNPs between AMA-positive and AMA-negative subgroups revealed neither significant allelic associations (Table 5) nor significant haplotype associations (Table 6), even when compared with early or late stages (Tables 5 and 6). Moreover, a comparison of 17 orthotopic liver transplantation (OLT) PBC cases and 291 non-OLT cases revealed no significant differences in allele frequencies (Table 5). In haplotype analysis, no statistical associations were found with OLT (Table 6).

Discussion

This study revealed that haplotype 1 (CGGA) was significantly associated with disease susceptibility in 273 AMA-positive PBC patients, as well as overall in all 308 PBC patients (p = 0.012) (data not shown). This finding is in agreement with the Caucasian study by Juran et al. [27], and thus constitutes a promising susceptibility gene candidate. However, since the precise function of *CTLA4* SNPs remains undefined, we cannot exclude the possibility that these SNPs may only be a linkage marker for a yet unidentified SNP within the *CTLA4* gene. Sequencing of the entire gene and assessing the functional role of these SNPs will be required.

SNP rs231775 associated with PBC is commonly referred to as 49AG in several studies [23,24,27,31,41]. Our finding corroborated a previous report [31], in which 49AG was not associated with susceptibility to PBC but there was a discrepancy in associ-

Table 5. Allele frequencies of *CTLA4* SNPs in AMA, histological or clinical disease progression, and OLT states.

SNP No.	Allele	AMA+* (n = 273)	AMA −* (n = 35)	p	Early* (n = 223)	Late* (n = 85)	p	Non-OLT* (n = 291)	OLT* (n = 17)	p
1	C	43.2	38.6	0.459	44.4	38.2	0.167	42.6	44.1	0.863
	T	56.8	61.4		55.6	61.8		57.4	55.9	
2	C	89.6	88.6	0.800	90.0	89.2	0.783	89.3	91.2	0.736
	T	10.4	11.4		10.0	10.8		10.7	8.8	
3	G	63.9	57.1	0.267	63.9	61.2	0.531	62.9	67.6	0.576
	A	36.1	42.9		36.1	38.8		37.1	32.4	
4	G	74.4	68.6	0.300	74.7	71.2	0.380	73.7	73.5	0.981
	A	25.6	31.4		25.3	28.8		26.3	26.5	
5	A	60.3	52.9	0.235	60.8	55.9	0.270	59.5	58.8	0.942
	G	39.7	47.1		39.2	44.1		40.5	41.2	

PBC, primary biliary cirrhosis; AMA, anti-mitochondrial antibodies; OLT, orthotopic liver transplantation; SNP, single nucleotide polymorphism; *, frequency (%).
p value was calculated by a χ^2-test 2 × 2 contingency table (df = 1).

Table 6. Comparison of *CTLA4* haplotype frequencies in AMA, histological or clinical disease progression, and OLT states.

Haplotype	SNPs No.				AMA+* (n = 546)	AMA −* (n = 70)	p	Early* (n = 446)	Late* (n = 170)	p	Non-OLT* (n = 582)	OLT* (n = 34)	p
	2	3	4	5									
1	C	G	G	A	60.1	52.8	0.245	60.5	55.9	0.292	59.3	58.8	0.959
2	C	A	A	G	25.5	30.0	0.415	25.1	28.2	0.430	26.1	23.5	0.738
3	T	A	G	G	10.3	10.0	0.947	10.3	10.0	0.909	10.3	8.8	0.781
4	C	G	G	G	3.5	4.3	0.720	3.1	4.7	0.346	3.4	5.9	0.458

PBC, primary biliary cirrhosis; AMA, anti-mitochondrial antibodies; OLT, orthotopic liver transplantation; SNP, single nucleotide polymorphism; *, proportion of indicated haplotype (%).
Values for n indicate two times the number of individuals since each person carries two haplotypes. p value was calculated by a χ^2-test 2 × 2 contingency table (df = 1).

ation with liver damage that might have arisen from the number of cases analyzed. 49AG also appears to affect cell surface expression of CTLA4 by CTLA4-driven down-regulation in response to T-cell activation [42]. This coding polymorphism is located in a signal peptide that is cleaved from the functional protein, and has been shown to affect glycosylation of the autoimmune susceptibility G allele, resulting in diminished processing efficiency and thus decreased trafficking to the cell surface [43]. It will be necessary to confirm the functional difference between patients with these SNPs and T-cell activation in a future study.

The rs3087243 SNP, also referred to as CT60, is located in the 3′ UTR of the *CTLA4* gene and reported to influence the production of the soluble isoform of CTLA4 (sCTLA4). The sCTLA4 mRNA encoded by the +CT60G-allele is produced at a reduced rate compared with that encoded by the A allele. As sCTLA4, which is secreted by resting T cells, is a suppressor of T-cell activation, it is conceivable that carriers of the +CT60G-allele allele may be more susceptible to autoimmune diseases [44]. Although studies from Canada and Italy found an association between PBC and the CT60 SNP [29,41], other studies have since failed to confirm this association [27,28], including ours.

In haplotype analysis, haplotype 1 contained all of the known SNP risk alleles that have been functionally determined in other disease studies. These include the C allele at −318, which has been found to affect the expression of CTLA4 mRNA cell surface expression [45], the minor G allele at 49AG, reported to reduce cell surface expression of CTLA4 [42], and the G allele of CT60, which affects the expression of the soluble form of the CTLA4 molecule, indicating the possibility that this haplotype might contribute to PBC susceptibility in the Japanese population.

Lastly, Juran et al. have suggested that CTLA4 plays a role in influencing AMA development as well as progression to OLT in PBC based on their haplotype analyses [27]. Our data revealed no statistical significance in regards to AMA development or disease progression to cirrhosis or OLT, possibly due to the number of patients showing AMA negativity and proceeding to OLT being too small to evaluate. Another consideration is that disease progression in Japanese patients might have a stronger association with positivity for anti-gp210 antibodies as a risk factor of progression to hepatic failure than *CTLA4* polymorphisms [46]. Further longitudinal follow-up studies in larger cohorts are required to resolve this critical question.

In conclusion, we found that *CTLA4* gene polymorphisms had a modest, but significant, association with susceptibility to PBC in the Japanese population and may share a common susceptibility haplotype with Caucasians. The connection between genetic variants and the function of the *CTLA4* gene remains to be addressed in future investigations.

Conflict of Interest

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

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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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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

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by the transcribed HCV genomes through detection by innate 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 15,000*g*. 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 20,000 *g* 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

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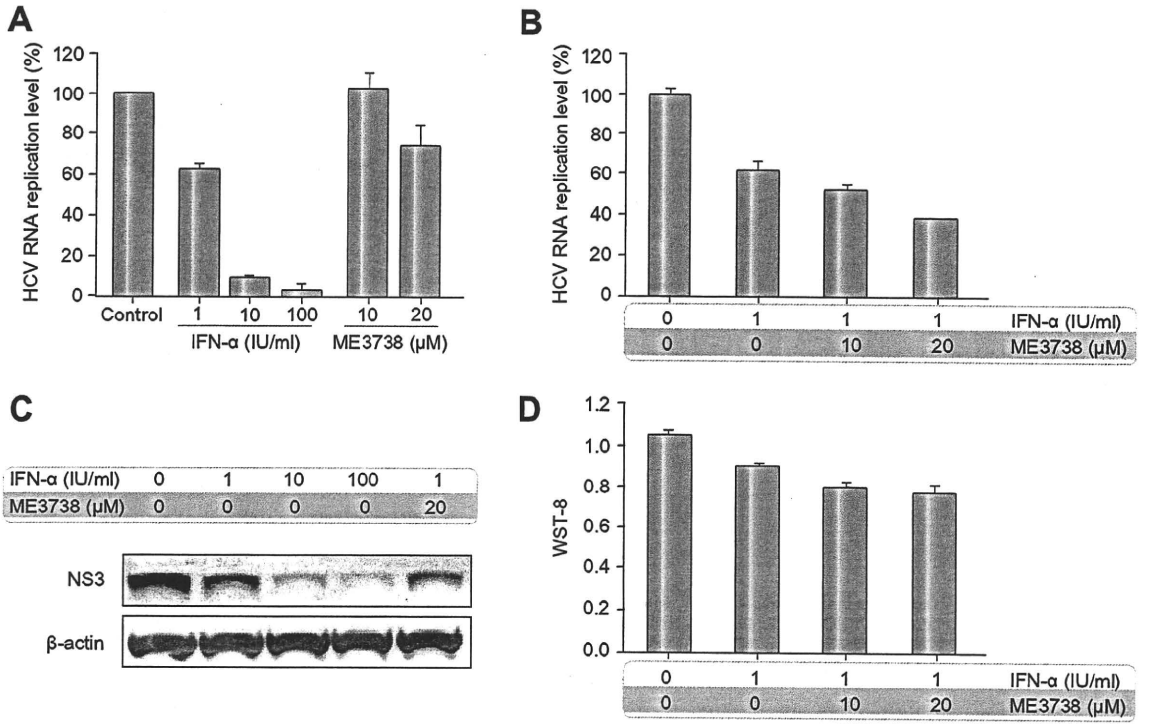

Fig. 1. Effects of ME3738 on HCV replication in the subgenomic HCV replicon, ORN/3-5B/KE cells. ORN/3-5B/KE cells were treated for 72 h with the indicated concentration of interferon (IFN)-α alone, ME3738 alone, or IFN-α plus ME3738. (A and B) Intracellular HCV RNA replication levels were determined 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 viabilities were 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

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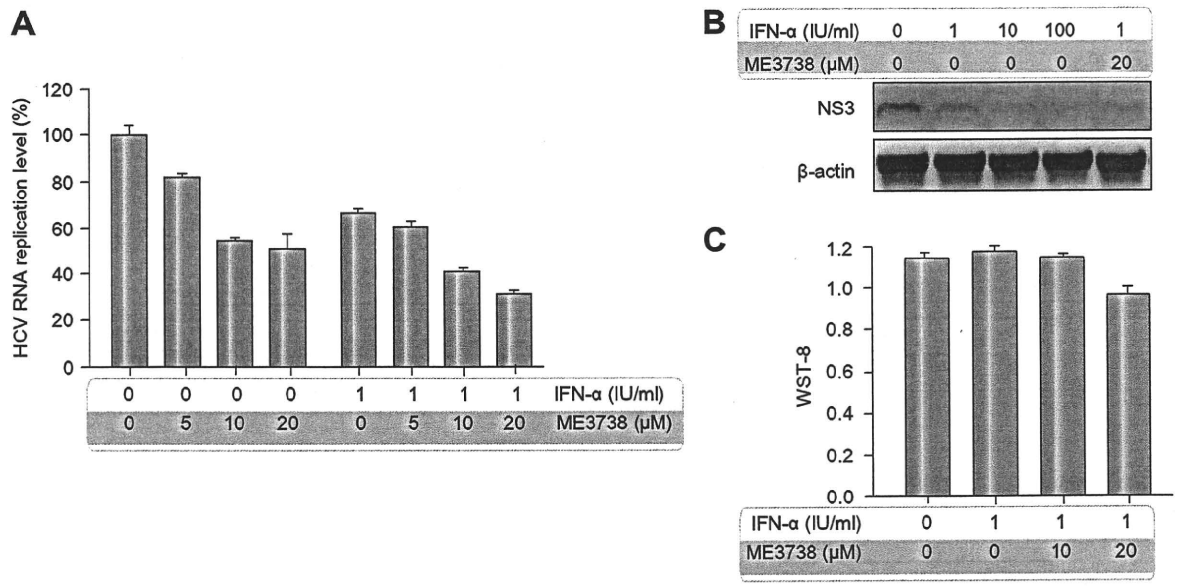

Fig. 2. Effects of ME3738 on HCV replication in the subgenomic HCV replicon, Con1 cells. Con1 cells were treated for 72 h with the indicated concentration of ME3738 alone or IFN-α plus ME3738. (A) Intracellular HCV RNA replication levels were determined via real-time PCR. (B) Cell lysates were analyzed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

ME3738/IFN-α-combination therapy (Fig. 4B). Since the level of HSA did not decrease in these treatments, it was concluded that 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.

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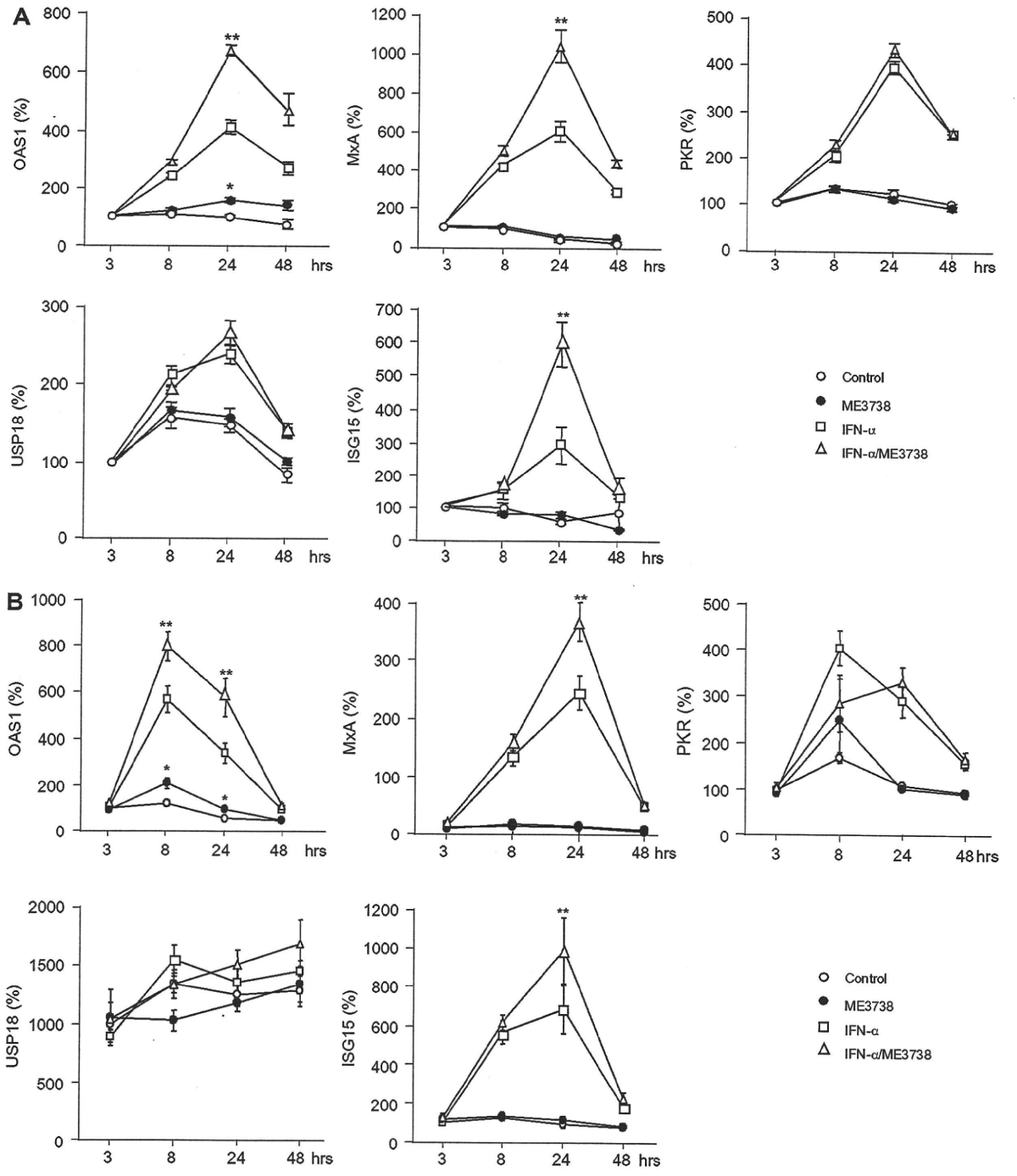

Fig. 3. Effects of ME3738 on the expression of interferon-stimulated genes. ORN/C-5B/KE cells (A) and Con1 cells (B) were treated with 20 μM of ME3738 and/or 1 IU/ml of interferon (IFN)-α for 48 h. Intracellular gene expression levels of oligoadenylate synthetase (OAS), myxovirus resistance protein A (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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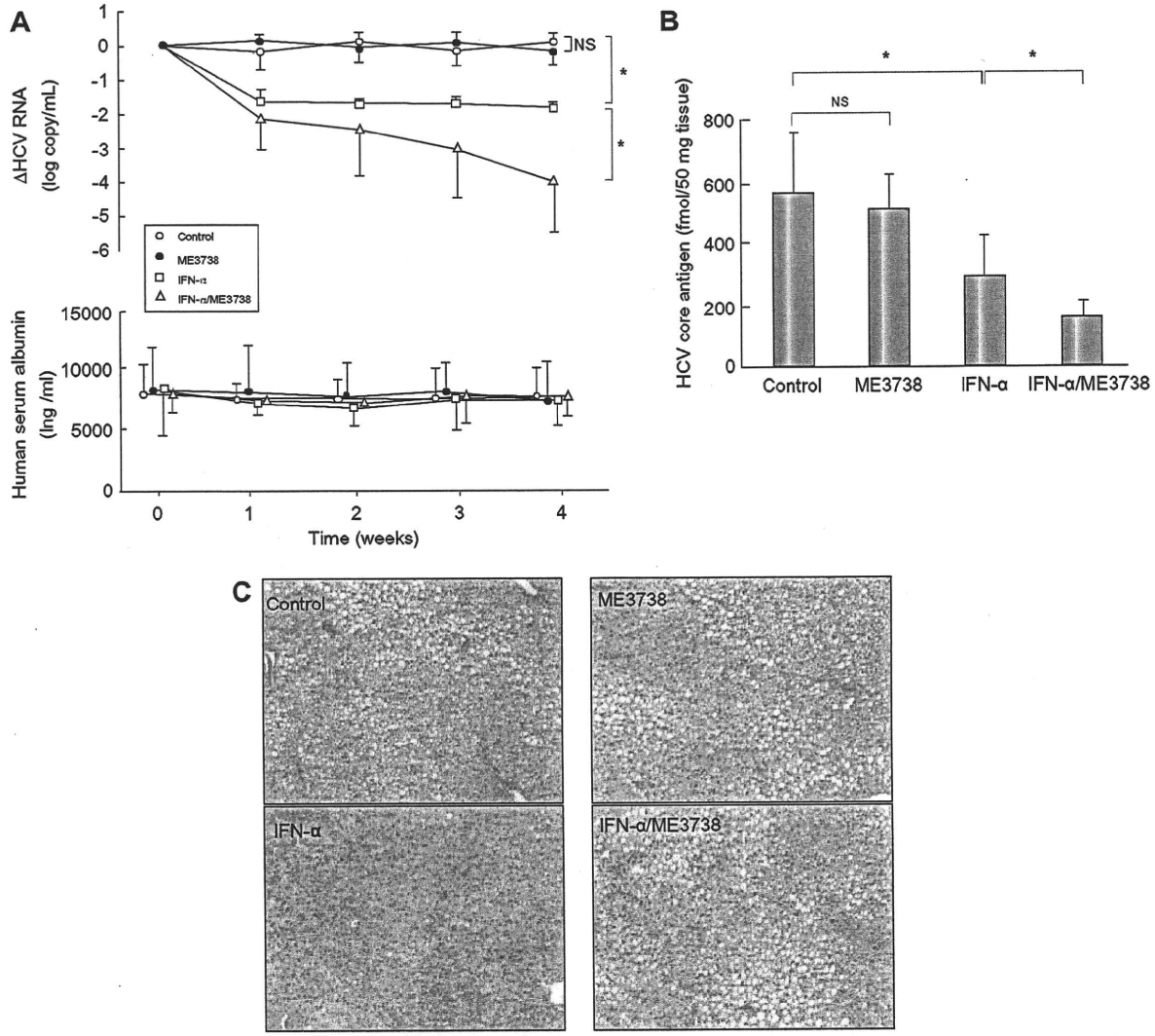

Fig. 4. ME3738 enhances the effect of IFN in mice with HCV infection. Mice were injected intravenously with 50 μl of HCV-positive human serum samples. Six weeks after HCV infection, mice were treated with ME3738 and/or interferon (IFN)-α for 4 weeks. (A) Mouse serum samples were obtained every week, and HCV RNA titer (upper panel) and human serum albumin concentration (lower panel) were analyzed. HCV core antigen was measured in the mouse livers after 4 weeks of treatment (B). 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.

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 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

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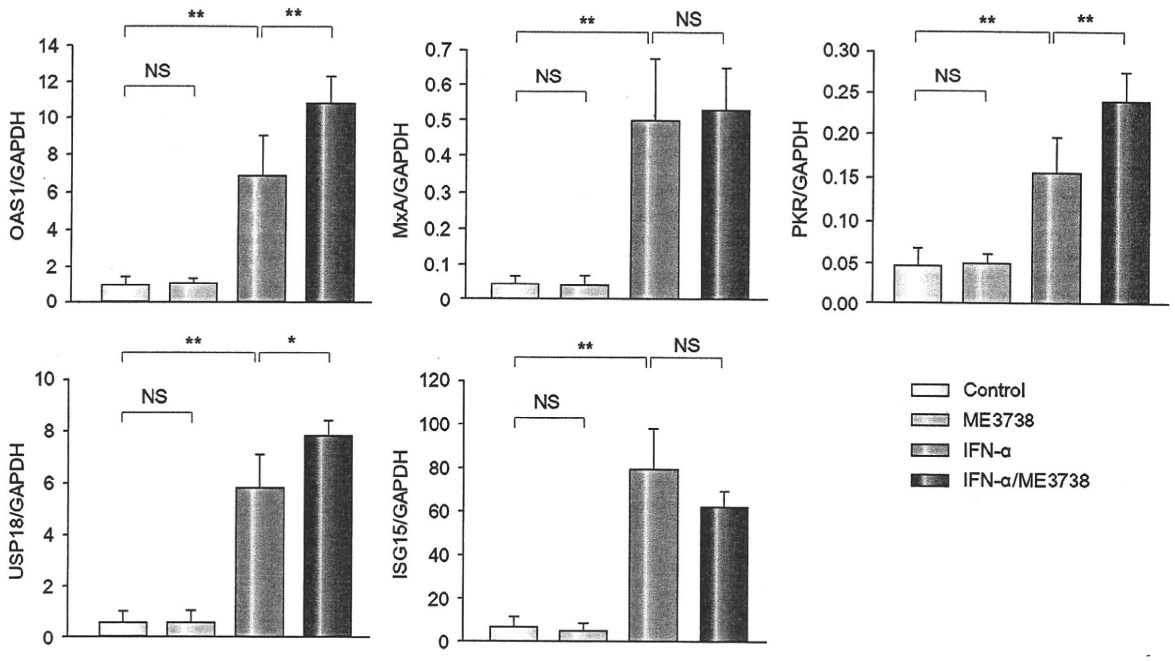

Fig. 5. Interferon stimulated genes expression in mouse liver samples. Mice were treated with or without 0.45% (w/w) ME3738 for 1 week and then given a single injection of 1500 IU/g IFN-α. Four hours after IFN-α injection, interferon stimulated gene expression in mouse livers were measured. RNA levels 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).

human studies should be conducted to develop an effective regimen for the treatment of patients with chronic hepatitis C.

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

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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.

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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-Vlbneo-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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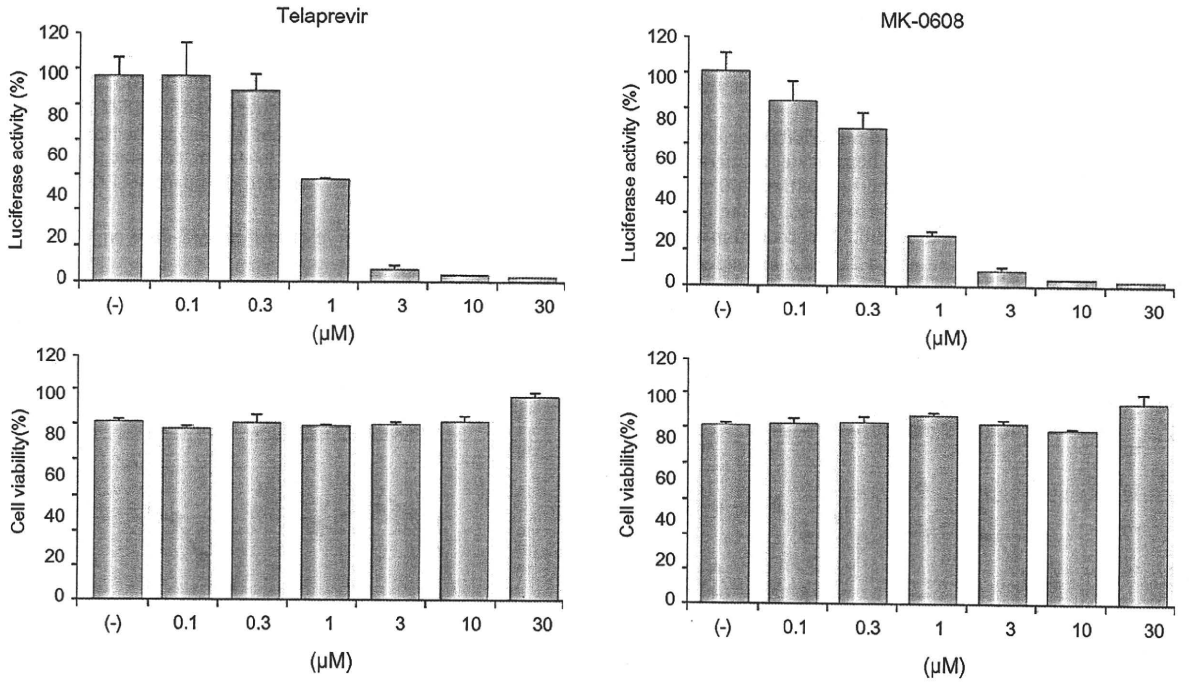

Fig. 1. *In vitro* analysis of susceptibility of HCV replicon cells to anti-HCV drugs. Huh7/Rep-Neo cells were treated for 72 h with the indicated concentrations of either telaprevir or MK-0608. Intracellular HCV RNA replication levels were determined as luciferase activities (upper panel), and expressed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

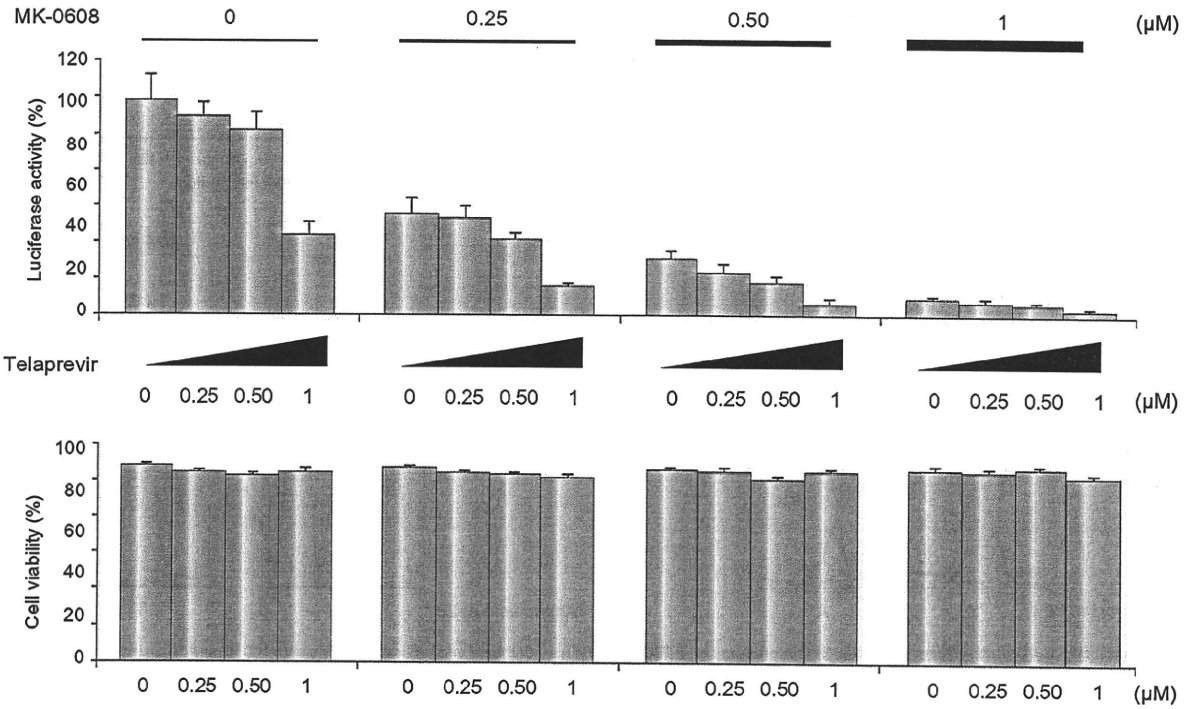

Fig. 2. *In vitro* analysis of susceptibility of HCV replicon cells to combination treatment with anti-HCV drugs. Huh7/Rep-Neo cells were treated for 72 h with the indicated concentration of MK-0608 plus telaprevir. Intracellular HCV RNA replication levels were determined as luciferase activities (upper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

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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

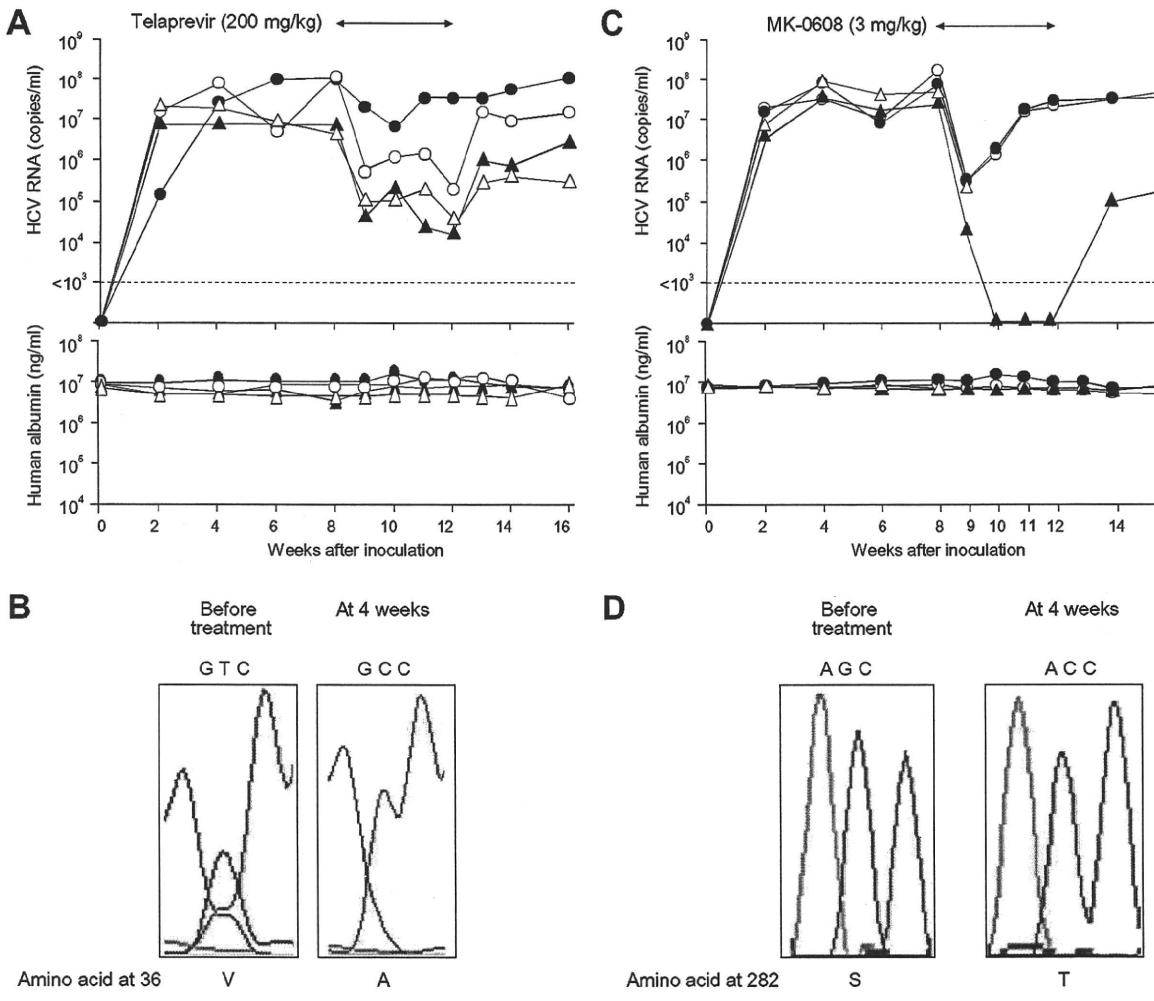

Fig. 3. Antiviral effects of either telaprevir or MK0608 monotherapy on HCV-infected mice. Mice were injected intravenously with 100 μl of HCV-positive human serum samples. Eight weeks after HCV infection, mice were treated with either 200 mg/kg of telaprevir (A) or 3 mg/kg of MK-0608 (C) for 4 weeks. 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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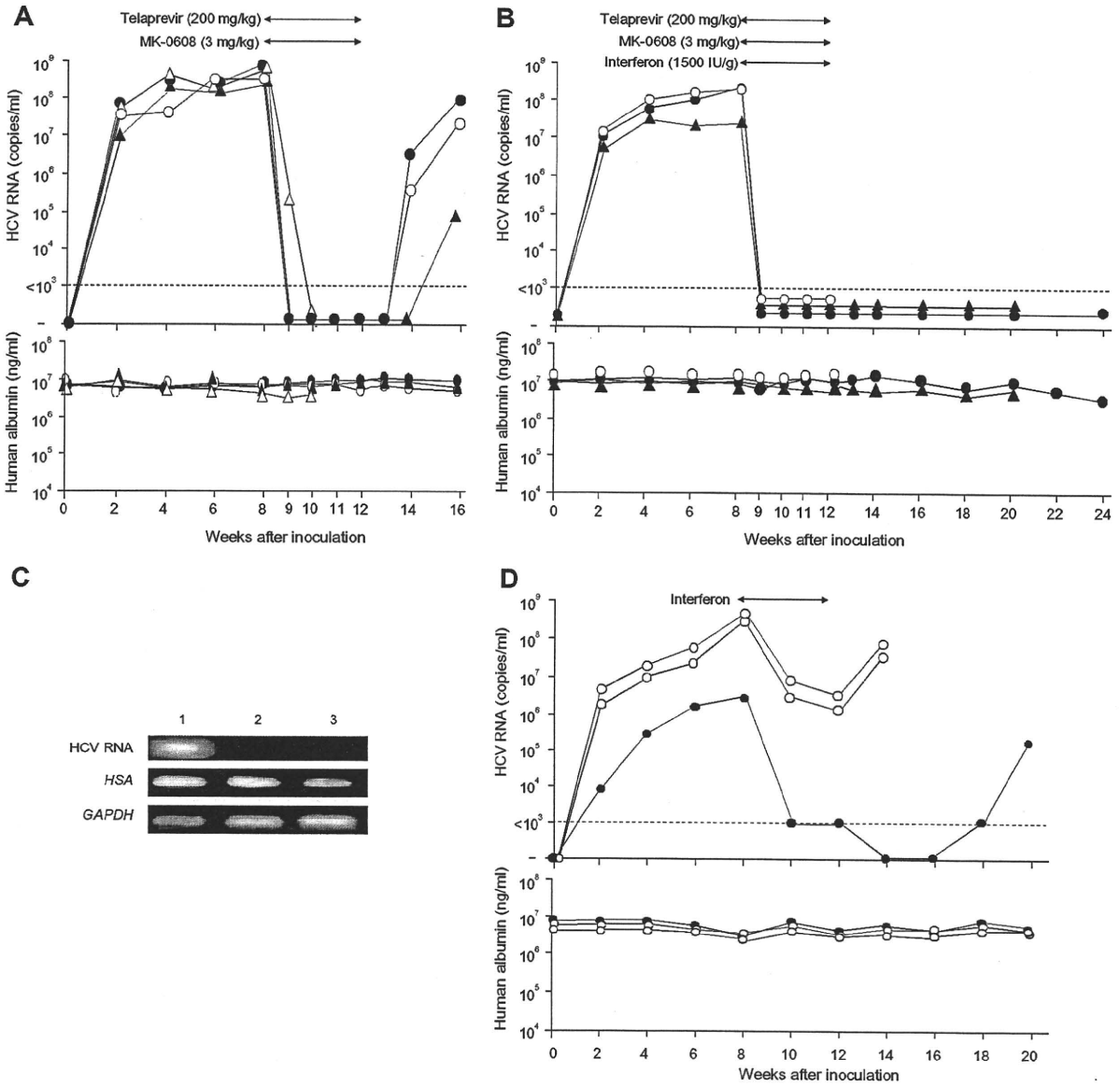

Fig. 4. Antiviral effect of combination treatment on HCV-infected mice. HCV-infected mice were treated with 200 mg/kg of telaprevir plus 3 mg/kg of MK-0608 without (A) or with (B) 1500 IU/g of human interferon-alpha for 4 weeks. Mice serum samples were obtained at the indicated times, and HCV RNA titer (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

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