Table 2 Rate of the ribavirin reduction or discontinuance due to adverse effects according to CL/F level

	No reduction	Dose reduction	Discontinuance	
			All cases	Cases due to severe anemia
$20 \leq CL/F$ ($n = 12$)	67% (8/12)	25% (3/12)	8% (1/12)	0
$15 \leq CL/F < 20$ ($n = 23$)	57% (13/23)	30% (7/23)	13% (3/23)	0
$10 \leq CL/F < 15$ ($n = 39$)	46% (18/39)	31% (12/39)	23% (9/39)	5% (2/39)
$CL/F < 10$ ($n = 42$)	33% (14/42)	40% (17/42)	26% (11/42)	5% (2/42)

$P = 0.031$ (Mantel–Haenszel χ^2-test).

Table 3 Minimum hemoglobin levels during PegIFN/ribavirin combination therapy according to CL/F level

	10 g/dL < Hb	8.5 < Hb ≤ 10 g/dL	Hb ≤ 8.5 g/dL
$20 \leq CL/F$ ($n = 12$)	92% (11/12)	12% (1/12)	0
$15 \leq CL/F < 20$ ($n = 23$)	83% (19/23)	17% (4/23)	0
$10 \leq CL/F < 15$ ($n = 39$)	72% (28/39)	23% (9/39)	5% (2/39)
$CL/F < 10$ ($n = 42$)	50% (21/42)	43% (18/42)	7% (3/42)

$P = 0.009$ (Mantel–Haenszel χ^2-test).

non-responders, 61% (23/38) in relapsers, and 58% (37/64) in naïve patients. The relationship between dose reduction or discontinuance of PegIFN and ribavirin and the SVR rate on ITT analysis is shown in Figure 1. Similar SVR rates were obtained in the groups without dose reduction of PegIFN and ribavirin (64%, 25/39) and with reduction of PegIFN and/or ribavirin (66%, 35/53); in detail, the SVR rate was 79% (11/14) in the group with reduction of only PegIFN, 55% (11/20) with reduction of only ribavirin, and 63% (12/19) with reduction of both PegIFN and ribavirin. In the group where both drugs were discontinued, the SVR rate was 25% (6/24), significantly lower than the group without reduction of both drugs ($P = 0.003$), and the group with reduction of PegIFN and/or ribavirin ($P = 0.001$).

CL/F and dose reduction or discontinuance of ribavirin

CL/F calculated for all patients showed a median of 12.6 L/h (range 4.5–27.9). At the start of the treatment, 36% (42/116) were under 10 L/h, 34% (39/116) were 10–15 L/h, 20% (23/116) were 15–20 L/h and 10% (12/116) were 20 L/h or more.

The rate of dose reduction or discontinuance of ribavirin is shown in Table 2 for different levels of CL/F. The rate of discontinuance of ribavirin in all cases was 8% (1/12) for the $CL/F \geq 20$, 13% (3/23) for the $15 \leq CL/F < 20$, 23% (9/39) for the $10 \leq CL/F < 15$, and 26% (11/42) for the $CL/F < 10$ group. Ribavirin did not have to be discontinued due to severe anemia among patients with $15 \leq CL/F$, but did for the 18% (2/11) of those with $CL/F < 10$ and 22% (2/9) of those with $10 \leq CL/F < 15$. The rate of reduction and discontinuance of ribavirin correlated significantly with the CL/F level.

CL/F and minimum hemoglobin level during treatment

To examine the relationship between anemia and the cessation of ribavirin in further detail, we evaluated the minimum hemoglobin level during treatment. Table 3 presents the different levels in relation to CL/F. The patients with minimum Hb ≤ 8.5 g/dL, the criterion for discontinuance of ribavirin, accounted for 7% (3/42) of the group of $CL/F < 10$, and 5% (2/39) of the group of $10 \leq CL/F < 15$. No patients of the group of $CL/F \geq 15$ showed minimum Hb ≤ 8.5 g/dL.

Early decline of Hb and progression of anemia during combination therapy

Following the initiation of combination therapy, the Hb concentration decreased rapidly until the end of four weeks. At the end of two weeks, Hb had decreased by 1.1 ± 1.0 g/dL among the patients without dose reduction of ribavirin ($n = 53$), 1.6 ± 1.2 g/dL among those with dose reduction ($n = 39$), and 1.8 ± 1.0 g/dL among

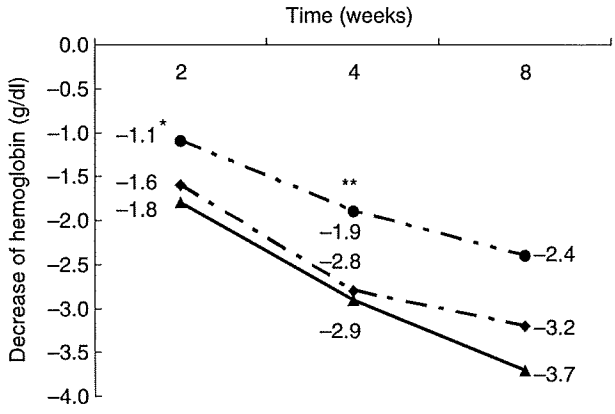

Figure 2 Course of ΔHb in the initial phase. (—··—), No reduction; (—·—·), reduction; (——), discontinuance. *Significantly different between patients with discontinuance and patients with no reduction ($P = 0.04$). **Significantly different between patients with discontinuance and patients with no reduction ($P = 0.008$), and between patients with discontinuance and patients with reduction ($P = 0.003$).

those who had discontinued ribavirin ($n = 24$). It was significantly different between the patients with no reduction and those with discontinuance of therapy ($P = 0.04$). At the end of four weeks, Hb had decreased by 1.9 ± 1.2 g/dL among the patients without dose reduction of ribavirin, 2.8 ± 1.2 g/dL among those with dose reduction, and 2.9 ± 1.2 g/dL among those who had discontinued ribavirin. Hb decline at the end of four weeks was significantly greater in the patients who had discontinued treatment and those who had reduced it, than in those with no reduction ($P = 0.008$, $P = 0.003$, respectively) (Fig. 2).

In this study, we selected the Hb decrease at the end of two weeks as the predictive factor for anemia progression. This is because the judgment of Hb decrease at the end of four weeks is too late to prevent progression of anemia or to perform appropriate counter-measures, such as the administration of epoetin or reduction of ribavirin. Next, we tried to use two borderlines of ΔHb: ΔHb 2.0 indicates a 2 g/dL Hb decrease at the end of two weeks and ΔHb 1.5 indicates a 1.5 g/dL Hb decrease. When ΔHb 2.0 was adopted, the rate of discontinuance of drugs was 31% (12/39) in the ΔHb ≥ 2.0 and 14% (11/76) in the ΔHb < 2.0. When ΔHb 1.5 was adopted, it was 23% (14/60) in the ΔHb ≥ 1.5 and 16% (9/55) in the ΔHb < 1.5. Comparison of the ΔHb 2.0 and ΔHb 1.5 standards showed the sensitivity to be 52% (12/23) and 61% (14/23), and the specificity to be 71% (65/92) and 50% (46/92), respectively. With respect to discontinuance due to anemia, both ΔHb 2.0 and ΔHb 1.5 gave 100% sensitivity (3/3), and the specificities were 68% (76/112) using ΔHb 2.0 and 49% (55/112) using ΔHb 1.5. We decided to adopt the standard of ΔHb 2 g/dL at the end of two weeks from the start of the pegylated IFN and ribavirin combination therapy as the predictive factor for anemia progression ("2 by 2" standard), which has been taken as a predictive factor for anemia in the IFN and ribavirin combination therapy.[25]

Applying the "2 by 2" standard to PegIFN plus ribavirin combination therapy, the rate of reduction or discontinuance of the ribavirin dose was examined with respect to the Hb decrease level (Table 4). Only one patient was excluded from this study, because the treatment was discontinued on the 11th day. In the group of ΔHb (the decrease in Hb concentration at two weeks from the baseline) ≥ 2 g/dL ($n = 39$), the doses were reduced for 18 patients (46%) and discontinued for 12 (31%), three of whom (8%) had severe anemia. For the group of ΔHb < 2 g/dL (76 patients), the doses were reduced for 21 patients (28%) and discontinued for 11 (14%); none due to severe anemia.

Early decline of Hb and minimum hemoglobin level during treatment

As in the case of ΔHb, we evaluated the minimum hemoglobin level during treatment, as shown in Figure 3. The patients with minimum Hb ≤ 8.5 g/dL accounted for 10% (4/39) of the group of ΔHb ≥ 2 g/dL, and there was no patient with minimum Hb ≤ 8.5 g/dL

Table 4 Rate of the ribavirin reduction or discontinuance due to adverse effects according to Hb decrease levels

	No reduction	Dose reduction	Discontinuance	
			All cases	Cases due to severe anemia
ΔHb < 2 g/dL ($n = 76$)	58% (44/76)	28% (21/76)	14% (11/76)	0
ΔHb ≥ 2 g/dL ($n = 39$)	23% (9/39)	46% (18/39)	31% (12/39)	8% (3/39)

$P = 0.004$ (Mantel–Haenszel χ^2-test).

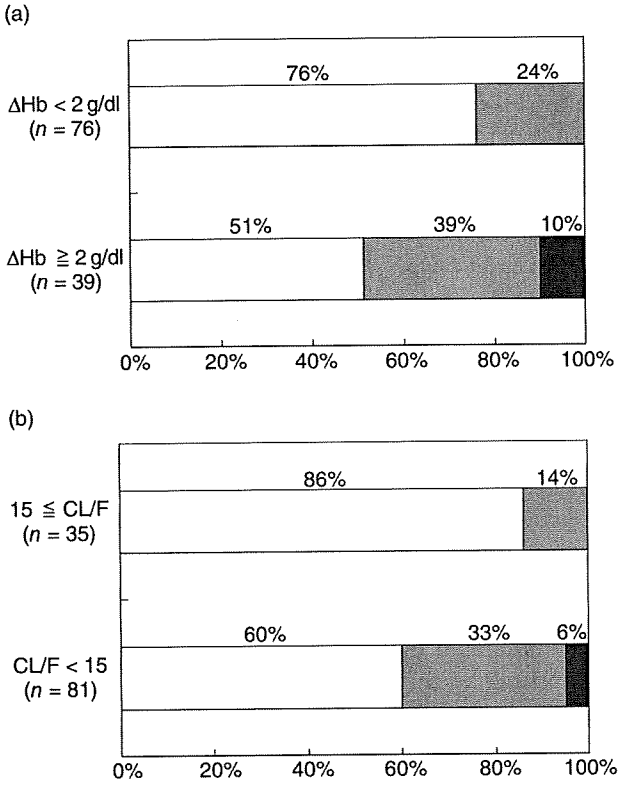

Figure 3 Minimum hemoglobin levels during PegIFN/ribavirin combination therapy. (□), 10 g/dL < minimum Hb; (▩), 8.5 < minimum Hb ≤ 10 g/dL; (■), minimum Hb ≤ 8.5 g/dL. (a) According to the "2 by 2" standard (Hb 2 g/dL decrease at two weeks from the baseline). $P = 0.009$ (Mantel–Haenszel χ^2-test). (b) according to CL/F levels. $P = 0.001$ (Mantel–Haenszel χ^2-test).

in the ΔHb < 2 g/dL group (Fig. 3a). The patients with minimum Hb ≤ 8.5 g/dL accounted for 6% (5/81) of the group of CL/F < 15, and there was no patient with minimum Hb ≤ 8.5 g/dL in the 15 ≤ CL/F group (Fig. 3b). The number of patients with minimum Hb ≤ 8.5 g/dL during PegIFN and ribavirin combination therapy according to "2 by 2" standard and CL/F levels is shown in Table 5. The patients with minimum Hb ≤ 8.5 g/dL were found only in the "2 by 2" standard-positive and low CL/F (<15) group (4/29, 14%).

Table 5 The number of patients with minimum hemoglobin ≤8.5 g/dL during PegIFN/ribavirin combination therapy according to "2 by 2" standard and CL/F levels

	ΔHb < 2 g/dL ($n = 76$)	ΔHb ≥ 2 g/dL ($n = 39$)
CL/F ≥ 15 ($n = 35$)	0/25	0/10
CL/F < 15 ($n = 80$)	0/51	4/29 (14%)

DISCUSSION

PREDICTION OF THE progression of anemia is necessary to decide whether drugs can be continued, with minimization of the disadvantages induced by anemia. Recently, CL/F has been used as a marker of progressing anemia that necessitates discontinuance of treatment. For example, if the patients have a low CL/F level, they should start treatment with a low ribavirin dose. In this study, we attempted to use the CL/F level measurement for our patients. To predict which patients might have to discontinue the treatment, the target range had to be CL/F < 15 because 6% of patients ($n = 5$) in this range showed minimum Hb ≤ 8.5 g/dL, which is the level at which ribavirin should be discontinued. No patients of the CL/F ≥15 group showed minimum Hb ≤ 8.5 g/dL. Our findings showed that 70% of the patients (81/116) with CL/F < 15 should be discriminated from the others (Table 3). In the same manner, using ΔHb as the marker, 34% of the target patients in the ΔHb ≥ 2 g/dL group were identified because 10% in this range showed minimum Hb ≤ 8.5 g/dL. No patients in the ΔHb < 2 g/dL group showed minimum Hb ≤ 8.5 g/dL. Compared to CL/F, ΔHb is considered to be more sensitive and convenient for identifying the high risk patients for whom treatment would need to be discontinued. Furthermore, the application of "2 by 2" standard in the group with low level of CL/F < 15 can be the most sensitive method for this (Table 5), since no patients with progression of anemia were found in the "2 by 2" standard-negative group with CL/F < 15.

In Japan, ribavirin doses are set at 600 mg for <60 kg, 800 mg for 60–80 kg, and 1000 mg for ≥80 kg, which are lower doses than those used in Europe and the USA. In this study, the mean ribavirin level at the start of treatment was 743 mg per day, while the AASLD practice guideline for genotype 1 hepatitis C is a daily dose of 1000 mg for body weight ≤ 75 kg and 1200 mg if >75 kg[26] In Japan, the use of lower doses is why fewer patients treated with PegIFN and ribavirin combination therapy are forced to discontinue the treatment due to severe anemia. Since the "2 by 2" model and/or CL/F can identify the patients who are prone to develop severe anemia, the other patients could be candidates for ribavirin dose-up strategies to raise SVR rates.

A considerable number of patients with chronic hepatitis C are over 60 years old in Japan (mean age is

around 55 years old),[27] although the mean age of this study was 50.6 years old. The number of aged patients with chronic hepatitis C is expected to increase in Europe and the USA, as well as in Japan. In IFN and ribavirin combination therapy, the discontinuance rate due to anemia was significantly higher in aged patients (≥60 years old, 21%) than in younger patients (<60 years old, 9%) ($P < 0.001$).[25] Earlier prediction of anemia is necessary to reduce the ribavirin dose in order to prevent the progression of severe anemia or to start epoetin alfa administration as needed, especially with aged patients. The "2 by 2" standard in PegIFN and ribavirin combination therapy should be a useful and convenient device for predicting the progress of anemia and treatment discontinuance in Europe and the USA, as well as in Japan.

CONCLUSION

IN CONCLUSION, THIS paper has shown that the SVR rate can be raised by preventing the discontinuance of ribavirin in PegIFN and ribavirin combination therapy. What is now needed is a prospective study of whether the early reduction of ribavirin in "2 by 2" standard-positive patients can improve the SVR rates, to ascertain the utility of the "2 by 2" standard in PegIFN and ribavirin combination therapy.

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Hepatitis C Virus Replication Is Inhibited by 22β-methoxyolean-12-ene-3β, 24(4β)-diol (ME3738) Through Enhancing Interferon-β

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A derivative of soyasapogenol, 22β-methoxyolean-12-ene-3β, 24(4β)-diol (ME3738), ameliorates liver injury induced by Concanavalin A in mice. We examined whether ME3738 has independent antiviral effects against hepatitis C virus (HCV) using an established HCV replication model that expresses the full-length genotype 1a HCV complementary DNA plasmid (pT7-flHCV-Rz) under the control of a replication-defective adenoviral vector expressing T7 polymerase. Hepatocellular carcinoma (HepG2) cells, human hepatoma (Huh7) cells, or monkey kidney (CV-1) cells were transfected with pT7-flHCV-Rz, and infected with adenoviral vector expressing T7 polymerase. ME3738 or interferon-α (IFN-α) was added thereafter and then protein and RNA were harvested from the cells at 9 days after infection. HCV-positive and HCV-negative strands were measured by real-time reverse-transcription polymerase chain reaction and HCV core protein expression was measured using an enzyme-linked immunosorbent assay. The messenger RNA levels of innate antiviral response-related genes were assessed using real-time reverse-transcription polymerase chain reaction. ME3738 dose-dependently reduced HCV-RNA and core protein in hepatocyte-derived cell lines. The antiviral effect was more pronounced in HepG2 than in Huh7 cells. ME3738 increased messenger RNA levels of interferon-β (IFN-β) and of IFN-stimulated genes (2′-5′ oligoadenylate synthetase, myxovirus resistance protein A [MxA]). Interferon-β knockdown by small interfering RNA abrogated the anti-HCV effect of ME3738. Moreover, the anti-HCV effects were synergistic when ME3738 was combined with IFN-α. *Conclusion:* ME3738 has antiviral effects against HCV. The enhancement of autocrine IFN-β suggests that ME3738 exerts antiviral action along the type I IFN pathway. This anti-HCV action by ME3738 was synergistically enhanced when combined with IFN-α. ME3738 might be a useful anti-HCV drug either with or without IFN-α. (HEPATOLOGY 2008;48:59-70.)

Abbreviations: Ad-T7pol, adenoviral vector expressing T7 polymerase; cDNA, complementary DNA; CV-1, African monkey kidney cell line; dsRNA, double-stranded RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCV, hepatitis C virus; HepG2, human hepatocellular carcinoma cell line; Huh7, human hepatoma cell line; IC_{90}, 90% inhibition concentration; IFN, interferon; IPS-1, interferon-β promoter stimulator 1; ISG, interferon stimulated gene; IRF-3, interferon regulatory factor 3; LacZ, β-galactosidase; ME3738, 22β-methoxyolean-12-ene-3β, 24(4β)-diol; mRNA, messenger RNA; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt; MxA, myxovirus resistance protein A; nt, nucleotide(s); OAS, oligoadenylate synthetase; PCR, polymerase chain reaction; PKR, protein kinase R; RIG-I, retinoic acid-inducible gene; RT, reverse-transcription; rTth, recombinant thermostable reverse transcriptase; siRNA, small interfering RNA; UTR, untranslated region.

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Hepatitis C virus (HCV) is a leading cause of chronic liver disease, including hepatitis, cirrhosis, and hepatocellular carcinoma.[1] Interferon (IFN) combined with ribavirin is the standard treatment for chronic HCV infection, although this combination more effectively eliminates genotype 2 rather than genotype 1 HCV.[2] Thus, an anti-HCV drug is required, particularly to combat infection with genotype 1.

The full-length HCV tissue culture model still has some limitations, not only for screening novel antiviral agents but also with respect to the ability to precisely characterize the antiviral effect of IFN, particularly against genotype 1 HCV infection. An infectious HCV production system has been established *in vitro* using genotype 2a in human hepatoma (Huh7) cells.[3,4] However, genotype 2a is not representative of the genotype 1 strains of HCV that are principally associated with liver disease worldwide. Although Yi et al.[5] established an infectious genotype 1a HCV production system, it produces small quantities of virus. The basis for HCV permissiveness in Huh7 cells has not been fully explained, but might relate to defects in endogenous IFN-β induction triggered by viral double-stranded RNA (dsRNA), and insufficient expression of Toll-like receptor 3 that recognizes dsRNA.[6] Therefore, interactions between host cellular proteins and HCV using systems based strictly on Huh7 cells might be confounded by the relative IFN-β deficiency in these cells.

We previously described cell-based HCV replication using a novel binary expression system in which mammalian cells were transfected with a T7 polymerase-driven full-length genotype 1a HCV complementary DNA (cDNA) plasmid and infected with a recombinant adenoviral vector encoding T7 polymerase.[7] This replication system is based on the genotype 1a isolate, H77, and it allows the evaluation of HCV replication in various cell lines, including Huh7. We used this model to identify new anti-HCV therapies with better profiles.

Triterpenoid saponins are natural sugar conjugates of triterpenes that possess various biological effects. Soyasaponins I and II extracted from Glycine max L. Merrill prevent liver injury and hyperlipidemia.[8] Furthermore, soyasaponin II has antiviral effects against herpes simplex virus, human cytomegalovirus, influenza virus, and human immunodeficiency virus.[9]

We demonstrated that soyasapogenol A, an aglycon of soyasaponin, ameliorates liver failure in a mouse model of Concanavalin A-induced liver injury.[10] We identified 22β-methoxyolean-12-ene-3β, 24(4β)-diol (ME3738), a derivative of soyasapogenol that ameliorates liver injury in several animal models of acute and chronic liver injury. If ME3738 exerts antiviral effects, especially against HCV, in addition to ameliorating liver damage, patients with HCV would derive more benefit. Therefore, the present study investigates the inhibitory effect of ME3738 against HCV replication, analyzes participating host factors, and measures the anti-HCV effect of ME3738 combined with IFN-α.

Materials and Methods

Reagents. The synthesis of ME3738 (Fig. 1) has been described.[11] We obtained ME3738 and IFN-α 2b from Meiji Seika (Yokohama, Japan) and from Schering-Plough (Kenilworth, NJ), respectively.

Cell Lines. We maintained hepatocellular carcinoma (HepG2) cells (American Type Culture Collection, Manassas, VA), human hepatoma (Huh7) cells (Dr. Robert Lanford, Southwest Foundation for Biomedical Research), and African green monkey kidney (CV-1) cells (American Type Culture Collection) in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

Plasmids and Transfection-Infection. The binary replication system can synthesize positive-strand and negative-strand HCV RNA, produce HCV protein, and generate quasispecies.[7,12,13] Briefly, we adapted a plasmid containing the infectious full-length genotype 1 cDNA sequence corresponding to the H77 prototype strain[14] at the 5′ and 3′ termini with the T7 promoter and a hepatitis delta virus ribozyme sequence, respectively, to yield pT7-flHCV-Rz. We transfected HepG2, Huh7, or CV-1 cells at 70% confluence on six-well plates with pT7-flHCV-Rz (3, 3, and 1 μg/well, respectively) using Lipofectamine (Invitrogen, Carlsbad, CA). To assess ME3738-induced inhibition of T7 polymerase activity, we transfected the pOS8 plasmid, which contains a T7 promoter flanking the β-galactosidase (LacZ) gene, into the cells. We delivered T7 polymerase to the HepG2, Huh7, and CV-1 cells using a replication-defective adenovirus vec-

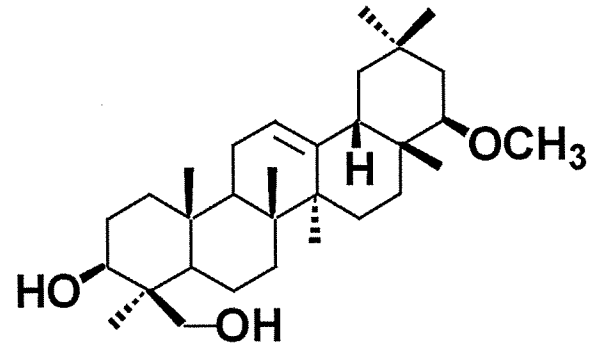


Fig. 1. Structure of ME3738. ME3738 is a derivative of soyasapogenol that ameliorates liver failure in mouse models of Concanavalin A (Con A)-induced liver injury.

tor (adenoviral vector expressing T7 polymerase [Ad-T7pol]) 24 hours after pT7-flHCV-Rz transfection. Control experiments included a replication-defective adenovirus vector lacking the T7 polymerase gene (Ad-Psi5; Harvard Gene Therapy Initiative Viral Vector Core Services, Boston, MA). We infected cells with the adenovirus vector at a multiplicity of infection of 10.

ME3738 and IFN-α Evaluation. We added ME3738 (0.1 to 10 μM) 3 hours after infection with Ad-T7pol. We also added IFN-α 2b (100 IU/mL) with or without ME3738 (0.1 μM) 3 hours after Ad-T7pol infection. We changed all media at day 1 after infection and every 2 days thereafter.

Cellular RNA Extraction. RNA extracted using TRIzol (Invitrogen) was digested twice for 4 hours each with deoxyribonuclease I using the DNA-free kit (Ambion, Austin, TX) following the manufacturer's protocol. Plasmid DNA was completely digested and removed from the RNA samples with deoxyribonuclease I because we did not detect any polymerase chain reaction (PCR) products. We quantified RNA by ultraviolet spectrum analysis.

Ribonuclease Protection Assay. We detected the HCV RNA-negative strand as described.[12] In brief, we generated antigenomic RNA by *in vitro* transcription using a sense-oriented [α-^{32}P] uridine triphosphate-labeled probe (corresponding to 98 nucleotides of the 3′ terminal HCV genome) and T7 polymerase from the vector pHCV-3′ T.[15] We generated transcripts using the RPA III kit (Ambion) according to the manufacturer's instructions.

Quantitative Real-Time Reverse-Transcription PCR. We quantified positive-strand and negative-strand HCV RNAs by real-time PCR using LightCycler technology (Roche Diagnostics, Mannheim, Germany) and SYBR green I dye (Roche Diagnostics) as described.[16] We synthesized complementary DNA from RNA (1 μg) in a mixture containing 5 U of recombinant thermostable reverse transcriptase (rTth) and 10 pM of the appropriate primer for reverse-transcription.[17,18] We detected positive-strand HCV RNA using the HCV-I antisense primer (5′-TGG ATG CAC GGT CTA CGA GAC CTC-3′, nucleotides [nt] 342-320 of the 5′ untranslated region [UTR]).[19] We detected negative-strand HCV RNA using the HCV-II sense primer (5′-CAC TCC CCT GTG AGG AAC T-3′, nt 38-56 of the 5′UTR)[19]. Positive-strand and negative-strand HCV PCR amplification proceeded using 2 μL of purified cDNA in a reaction mixture containing 1 μL of LightCycler Fast Start DNA Master SYBR Green I, 4 mM of $MgCl_2$, 5 pM of the antisense primer KY78 (5′-CTC GCA AGC ACC CTA TCA GGC AGT-3′; nt 311-288 of the 5′UTR), and 5 pM of the sense primer KY80 (5′-GCA GAA AGC GTC TAG CCA TGG CGT-3′; nt 68-91 of the 5′UTR). The PCR amplification consisted of initial denaturation for 10 minutes at 95°C, followed by 50 cycles of 15 seconds at 95°C, 5 seconds at 70°C, and 15 seconds at 72°C.

We quantified cellular messenger RNA (mRNA) by reverse-transcription with the same amount of RNA that was used for HCV-positive and HCV-negative strand analysis, and the oligonucleotide $d(T)_{16}$ primer under standard conditions.[20] For real-time PCR amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), IFN-β, IFN-α, 2′-5′ oligoadenylate synthetase (OAS), myxovirus resistance protein A (MxA), and IFN regulatory factor 3 (IRF-3), we included commercial primer sets for each (Roche Search LC, Mannheim, Germany). For real-time PCR amplification of protein kinase R (PKR), we applied the forward (5′-AGC ACA CTC GCT TCT GAA TC-3′) and reverse (5′-CTG GTC TCA GGA TCA TAA TC-3′) primers under the following conditions: 10 seconds at 95°C, 10 seconds at 58°C, and 15 seconds at 72°C.[20] We included specific primers for retinoic acid-inducible gene I (RIG-I: forward primer, 5′-GTG CAA AGC CTT GGC ATG T-3′ and reverse primer, 5′-TGG CTT GGG ATG TGG TCT ACT C-3′), and IFN-β promoter stimulator 1 (IPS-1: forward primer, 5′-GCA ATG TGG ATG TTG TAG AG-3′ and reverse primer, 5′-CTG AAG GGT ATT GAA GAG ATG-3′) under the following conditions: 10 seconds at 95°C, 10 seconds at 60°C, and 6 seconds at 72°C. For real-time PCR amplification of LacZ, we included sense (5′-GCC TGC GAT GTC GGT TTC CGC GAG G-3′) and antisense (5′-GCC AGC GCG GAT CAT CGG TCA GAC G-3′) primers under the following conditions: 10 seconds at 95°C, 10 seconds at 68°C, and 16 seconds at 72°C.[21]

We quantified DNA by measuring SYBR green I dye incorporation into PCR products at 530 nm according to the manufacturer's instructions. We generated a standard curve for HCV using a PCR product corresponding to nt 38-342 of the 5′UTR. At the end of each run, we generated a DNA melting curve to control for sample homogeneity and quality. We electroporated a subset of samples and sequenced them to confirm the identity of the amplified PCR product. Data are expressed as the copy numbers of HCV RNA or cellular mRNA per molecule of GAPDH mRNA.

Enzyme-Linked Immunosorbent Assay for HCV Core Antigen. We adjusted cell culture lysates to 0.2 mg/mL. We measured concentrations of HCV core antigen using the HCV core protein enzyme-linked immunosorbent assay (ELISA) kit (Ortho-Clinical Diagnostics, Raritan, NJ) following the manufacturer's instructions.[22]

Core ELISA data are expressed as femtomoles of HCV core antigen per microgram of total protein. The lower level of detection for this assay was below 1.5 pg/mL.

MTS Assay. We assessed reagent cytotoxicity using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay with the CellTiter 96 Aqueous One Solution cell proliferation assay (Promega, Madison, WI), according to the manufacturer's instructions.

RNA Interference and Neutralizing IFN-β by Antibody. We transfected cells with three small interfering RNAs (siRNAs) targeting IFN-β using Lipofectamine RNAiMax (Invitrogen). The final siRNA concentration was 20 nM at 1 day before transfection with pT7-flHCV-Rz. The target sequences of the three IFN-β siRNAs were AAU CCA AGC AAG UUG UAG CUC AUG G (HSS105232), AAG CCU CCC AUU CAA UUG CCA GG (HSS105233), and AUU UGG AGG AGA CAC UUG UUG GUC A (HSS105234), respectively. We purchased the IFN-β siRNAs and negative control siRNA from Invitrogen.

We used mouse antihuman IFN-β antibody (R&D Systems, Minneapolis, MN) or mouse isotype control antibody (R&D Systems) to neutralize IFN-β in cell culture supernatants. We added 1 μg/mL of each antibody to the supernatant after infection with Ad-T7pol, and then incubated it until day 3.

Statistical Analysis. We statistically analyzed values using SPSS 14.0 software (SPSS, Tokyo, Japan). We compared differences in mean values using the Mann-Whitney U-test.

Results

Replication-Defective Adenoviral Vectors Replicated HCV RNA in HepG2 Cells. Previously, we refined a full-length HCV replication system in the hepatocyte-derived and non-hepatocyte-derived cell lines, Huh7 and CV-1, respectively.[7] The Huh7 cell line or its derivatives are used in many HCV replication systems, such as replicon models. However, because of the relative IFN-β deficiency of Huh7 cell lines,[6] interactions between the host cellular proteins and HCV in the Huh7 cell line might differ from those *in vivo*. Therefore, we examined whether or not HCV replicates in another hepatocyte-derived cell line. HepG2 cells were transfected with pT7-flHCV-Rz and then infected with Ad-T7pol. The ribonuclease protection assay detected HCV-negative strands in HepG2 (Fig. 2A), Huh7, and CV-1 (Fig. 2B) cells. The parental replication-incompetent vector Ad-Psi5 served as an adenoviral vector control. Negative-strand HCV RNA was undetectable in HepG2, Huh7,

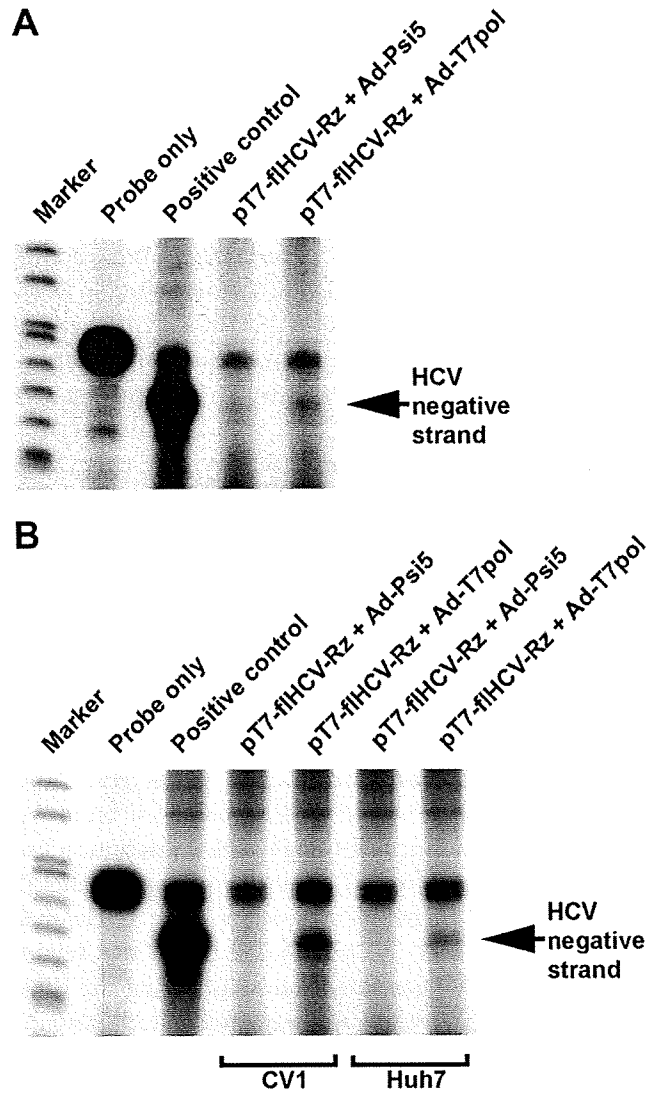
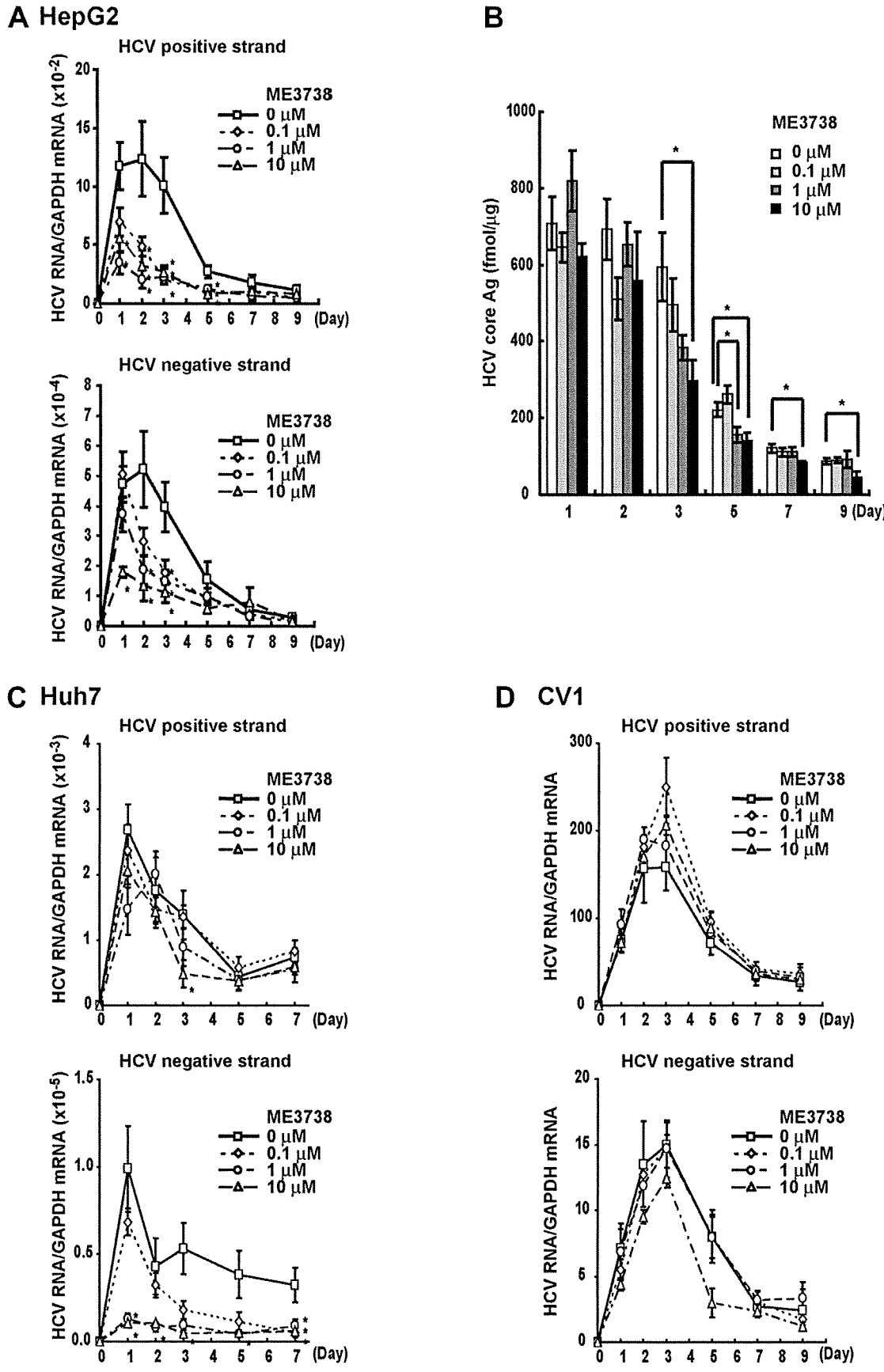


Fig. 2. Synthesis of HCV RNA-negative strand. After transfection with pT7-flHCV-Rz plasmid, cells were infected with adenoviral vectors expressing recombinant adeno-T7 polymerase (Ad-T7pol) or with adenoviral vectors lacking T7 polymerase gene (Ad-Psi5) at multiplicity of infection (MOI) of 10. Ribonuclease protection assays detected HCV-negative strands in (A) HepG2, and (B) Huh7 and CV-1cells.

Fig. 3. Inhibitory effects of ME3738 on HCV replication in hepatocyte-derived cell lines. (A) Real-time reverse-transcription PCR (RT-PCR) quantified HCV-positive and HCV-negative strands over time in cells cultured with or without ME3738. Levels of both HCV RNA strands were significantly and dose-dependently decreased by ME3738 in HepG2 cells. Data indicate means ± standard error (SE) for six replicates (*$P < 0.05$). (B) Decrease of HCV core protein after HCV RNA decline determined by enzyme-linked immunosorbent assay (ELISA). Data indicate means ± SE for four replicates (*$P < 0.05$). (C) ME3738 significantly decreased HCV-positive strand only at day 3 in Huh7 cells, but not to the extent found in HepG2 cells. ME3738 more significantly reduced HCV RNA-negative, than HCV RNA-positive strands in Huh7 cells. Data indicate means ± SE for six replicates (*$P < 0.05$). (D) Levels of neither HCV RNA strand significantly decreased in CV-1 cells, suggesting that anti-HCV effects of ME3738 are specific to hepatocyte-derived cell lines. Data indicate means ± SE for three replicates.


A HepG2
HCV positive strand
HCV RNA/GAPDH mRNA (x10-2)
ME3738
0 μM
0.1 μM
1 μM
10 μM
(Day)
HCV negative strand
HCV RNA/GAPDH mRNA (x10-4)
B
HCV core Ag (fmol/μg)
C Huh7
HCV positive strand
HCV RNA/GAPDH mRNA (x10-3)
HCV negative strand
HCV RNA/GAPDH mRNA (x10-5)
D CV1
HCV positive strand
HCV RNA/GAPDH mRNA
HCV negative strand

and CV-1 cells transfected with the control Ad-Psi5 vector (Fig. 2A,B).

ME3738 Inhibited HCV Expression. To assess the inhibitory effects of ME3738 on HCV replication, we cultured cells with ME3738 (0, 0.1, 1, and 10 μM) after Ad-T7pol infection.

Levels of HCV RNA-positive (Fig. 3A) and HCV RNA-negative strands in HepG2 cells were significantly and dose-dependently decreased by ME3738 from days 1 to 5 and from days 1 to 3, respectively. The concentration of ME3738 required for 50% inhibition (IC_{50}) of HCV RNA-positive strands calculated from the dose-response data obtained on day 2 with ME3738 was 0.03 μM. ME3738 significantly and dose-dependently decreased levels of HCV core protein in HepG2 cells on days 3 to 9; that is, after the HCV RNA level decreased (Fig. 3B).

ME3738 significantly and dose-dependently decreased HCV RNA-positive strands in Huh7 cells (Fig. 3C) at day 3. The dose-dependent effect of ME3738 was even more pronounced on the level of HCV RNA-negative, than HCV RNA-positive strands in Huh7 cells, and was detectable from days 1 to 9. Whereas a significant decrease in HCV RNA levels was associated with ME3738 in HepG2 and Huh7 cells, no such decrease was evident in CV-1 cells (Fig. 3D). These findings suggest that ME3738 has antiviral effects against HCV in hepatocyte-derived cells and that the effect is more pronounced in HepG2, than in Huh7 cells.

To confirm that the reduction in HCV RNA levels induced by ME3738 in HepG2 cells was not due to a nonspecific response such as inhibited T7 polymerase activity, we transfected the plasmid OS8 into HepG2 cells and then measured LacZ mRNA levels in the presence of various doses of ME3738. ME3738 did not significantly reduce LacZ mRNA expression in HepG2 cells (Supplementary Fig. 1A). In addition, the MTS assay showed that ME3738 was not significantly cytotoxic to HepG2 cells (Supplementary Fig. 1B).

Huh7 cells have impaired Toll-like receptor 3 and dsRNA signaling, and thus, limited induction of IFN-stimulated genes (ISGs).[6,23,24] Because ME3738 exerts more powerful antiviral effects against HCV in HepG2 than in Huh7 cells, we examined the signaling pathway of IFN and ISGs.

IFN-β and ISG mRNA Expression in HepG2 and Huh7 Cells. The induction of type I IFNs represents an early protective response to many viral infections in mammalian cells. The induction of IFN-β in particular represents the immediate response of cells to viral infection, and precedes the transcription of most IFN-α species.[25] ME3738 inhibited HCV replication soon after Ad-T7pol infection in hepatocyte-derived cell lines. Because the inhibitory effects against HCV replication by ME3738 were the most rapid and robust in HepG2 cells, we examined IFN-β mRNA expression in our replication model using these cells. After expression with HCV without ME3738, high levels of IFN-β mRNA persisted for 3 days and diminished thereafter in HepG2 cells (Fig. 4A). The IFN-β mRNA level was increased up to 340-fold compared with the basal level. At day 1, which is the earliest time point after Ad-T7pol infection, ME3738 dose-dependently increased IFN-β mRNA expression in HepG2 cells. To further determine whether the IFN-β mRNA expression enhanced by ME3738 depends on an antiviral response against HCV replication, we transfected the plasmid OS8 into HepG2 cells with or without ME3738, and we measured IFN-β mRNA levels (Fig. 4B). Although the IFN-β mRNA level in the cells cultured without ME3738 was increased up to only 4.5-fold compared with the basal level, it was 1.3% of that in cells expressing HCV. Moreover, ME3738 did not enhance IFN-β mRNA expression in cells transfected with pOS8 (Fig. 4B). The IFN-β enhancement was more remarkable in HepG2 than in Huh7 cells (Fig. 4B). The IFN-β mRNA levels in Huh7 cells without ME3738 were increased by only up to three-fold compared with the 340-fold increase in HepG2 cells. Moreover, the level of IFN-α mRNA was not altered in HepG2 and Huh7 cell lines cultured with ME3738 (Supplementary Fig. 2).

Because ME3738 significantly enhanced the IFN-β expression activated by HCV replication at the earliest time point, we considered whether ISGs are also increased following IFN-β enhancement. In HepG2 cells treated without ME3738, 2′-5′ OAS, MxA, and PKR mRNA were also induced by HCV RNA replication at day 2. The expression of both 2′-5′ OAS and MxA mRNA in HepG2 cells was also significantly and dose-dependently enhanced by ME3738 at day 2, but not that of PKR mRNA (Fig. 4C). These data demonstrated that ME3738 enhanced the induction of endogenous IFN-β and ISGs triggered by the dsRNA of HCV replication. Like the unremarkable enhancement of IFN-β mRNA expression by ME3738 (Fig. 4B), the mRNA expression of the ISGs, 2′-5′ OAS, MxA, and PKR, was not enhanced in Huh7 cells (Fig. 4D) compared with that in HepG2 cells. These findings suggested that the differences in the anti-HCV effects in HepG2 and Huh7 cells correlate with cell-specific variations in IFN-β mRNA expression.

Intracellular dsRNA produced during viral replication is recognized by RIG-I,[26,27] then IPS-1 activates IRF-3, which subsequently induces IFN-β transcription.[28,29] We therefore examined these molecules, and measured each mRNA level by quantitative real-time RT (reverse-transcription)-PCR. However, ME3738 had no effect on

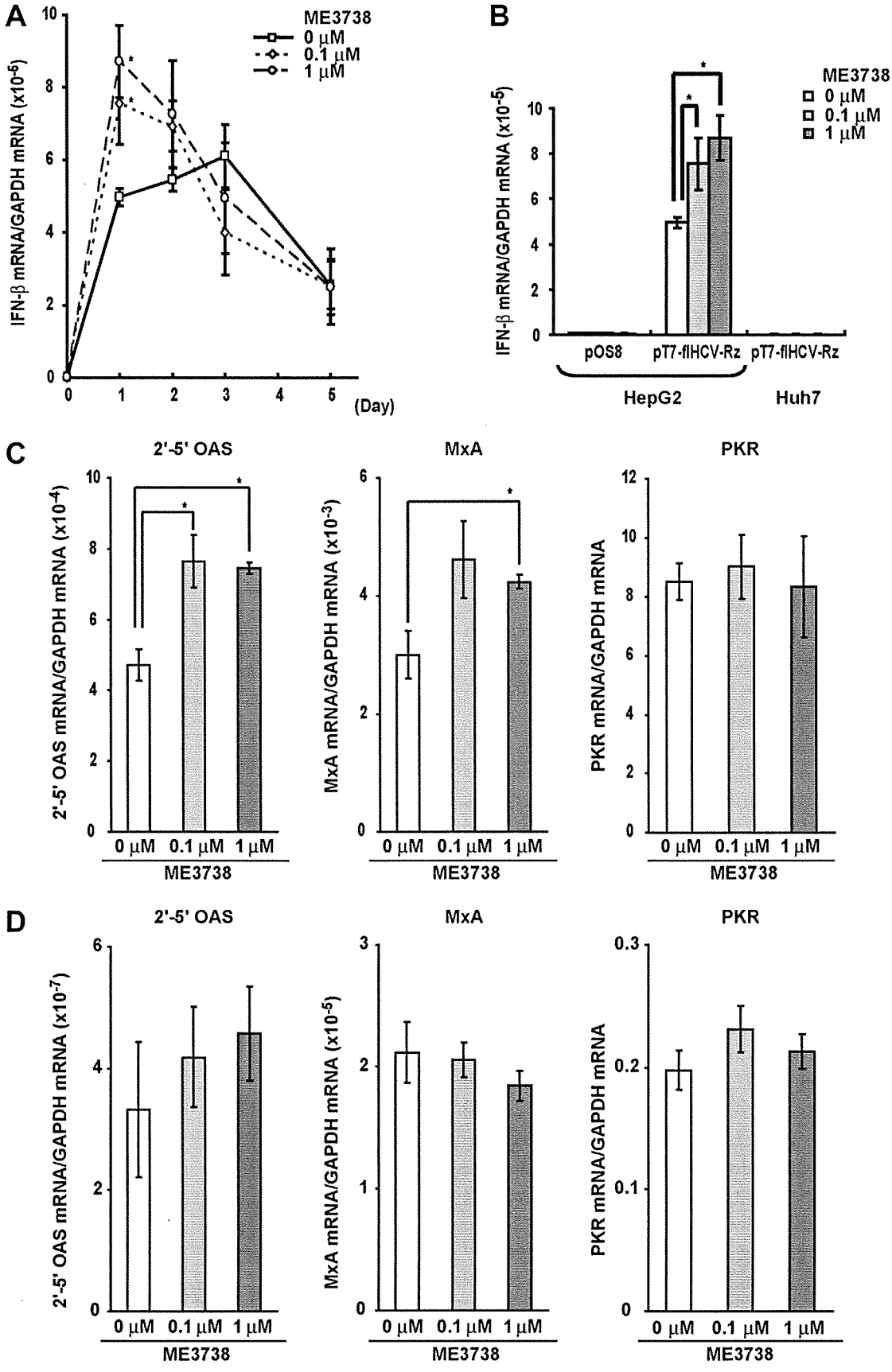


Fig. 4. ME3738 enhances IFN-β and ISG expression in HepG2 cells. (A) Time course of IFN-β mRNA assessed by real-time reverse-transcription PCR (RT-PCR). ME3738 more significantly and dose-dependently increased IFN-β mRNA expression in HepG2 cells at day 1 (earliest time point after Ad-T7pol infection). (B) ME3738 induced little or no IFN-β mRNA in HepG2 cells transfected with plasmid OS8 instead of pT7-flHCV-Rz, or in Huh7 cells transfected with pT7-flHCV-Rz, respectively. (C) ME3738 upregulated 2′-5′ OAS and MxA, but not PKR mRNA in HepG2 cells. (D) ME3738 did not enhance either 2′-5′ OAS, MxA, or PKR mRNA expression in Huh7 cells. Data from HepG2 or Huh7 cells indicate means ± standard error (SE) for four and six replicates, respectively (*$P < 0.05$).

RIG-I, IPS-1, and IRF-3 mRNA expression at day 1, or at the earliest time point (data not shown). ME3738 seemed to enhance IFN-β expression triggered by HCV replication without augmentation by receptor, adaptor protein, and transcription factor mRNA expression.

Enhanced IFN-β Participates in Anti-HCV Effects of ME3738 in HepG2 Cells. We applied RNA interference to knock down IFN-β expression and determine whether ME3738 inhibits HCV replication through IFN-β enhancement. Cells transfected with IFN-β-specific siRNA before pT7-flHCV-Rz, but not with scrambled control siRNA, caused an 85% reduction in the IFN-β mRNA level triggered by HCV replication in the presence or absence of ME3738 (Fig. 5A). Furthermore, ME3738 exerted anti-HCV effects in HepG2 cells transfected with scrambled control siRNA, but not with IFN-β siRNA (Fig. 5B). We also included a neutralizing antibody to IFN-β in cell supernatants. The anti-HCV effect of ME3738 was also diminished on day 3 in cells incubated with the neutralizing antibody compared with the control antibody (Fig. 5C). These data indicate that ME3738 requires enhanced IFN-β expression to exert anti-HCV effects in HepG2 cells.

ME3738 and IFN-α Synergistically Inhibit HCV Replication. The combination of IFN and ribavirin is the current standard therapy for infection with chronic hepatitis C.[2] Because we found that ME3738 inhibited HCV replication through IFN-β enhancement, we postulated that ME3738 could be combined with IFN like ribavirin.

Our previous study showed that 1,000 IU/mL of IFN-α inhibits HCV replication from day 2.[7] However, a low dose of IFN (100 IU/mL) alone is insufficient to inhibit HCV replication. To evaluate whether ME3738 augments the anti-HCV activity of IFN-α, HepG2 cells were infected with Ad-T7pol and then incubated with suboptimal doses of ME3738 (0.1 μM) and IFN-α (100 IU/mL) (Fig. 6A). The results showed that HCV replication was considerably reduced by the combination compared with that by either ME3738 or IFN alone. We examined whether the effect of this combination was synergistic or additive using the isobologram method.[30,31] The combination of ME3738 (0.1 μM) and IFN-α (100 IU/mL) inhibited about 90% of HCV RNA replication 2 days after Ad-T7pol infection. The amounts of IFN-α and ME3738 to induce 90% inhibition (IC_{90}) were 1,792 IU/mL and 1.06 μM, respectively. These data were used to generate isoboles, which demonstrated 90% inhibition of HCV RNA replication, and the 90% isobole revealed that the anti-HCV action of ME3738 and IFN-α was synergistic (Fig. 6B).

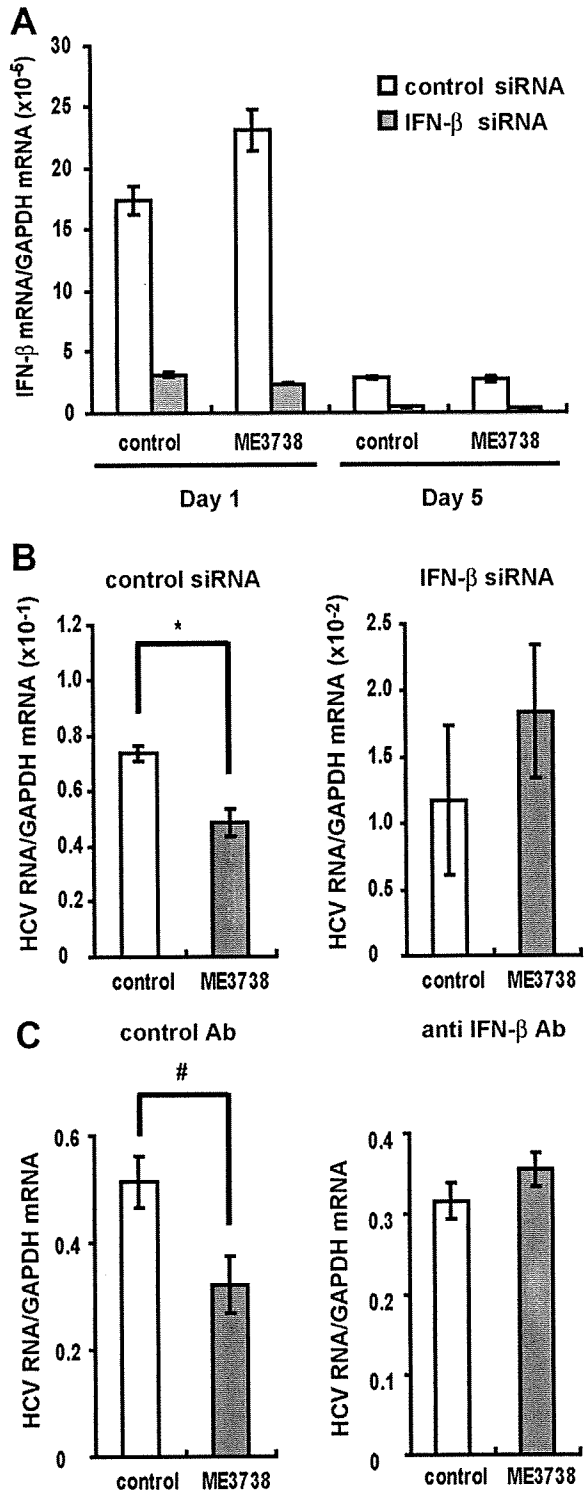


Fig. 5. Anti-HCV effects of ME3738 abrogated in HepG2 cells transfected with IFN-β siRNA. HepG2 cells were transfected with siRNAs targeting IFN-β before HCV expression. Data indicate means ± standard error (SE) for four replicates. (A) Transfection with IFN-β-specific siRNA caused reproducible 85% reductions in IFN-β mRNA levels with or without ME3738. (B) Transfection with IFN-β siRNA reduces anti-HCV effects of ME3738, indicating that enhanced IFN-β expression correlates with anti-HCV effects of ME3738 (*$P < 0.05$). (C) Neutralizing antibody to IFN-β (anti IFN-β Ab) or control antibody (control Ab) added to cell culture supernatants after infection with Ad-T7pol diminished anti-HCV effects of ME3738 compared with control Ab (#$P = 0.061$).

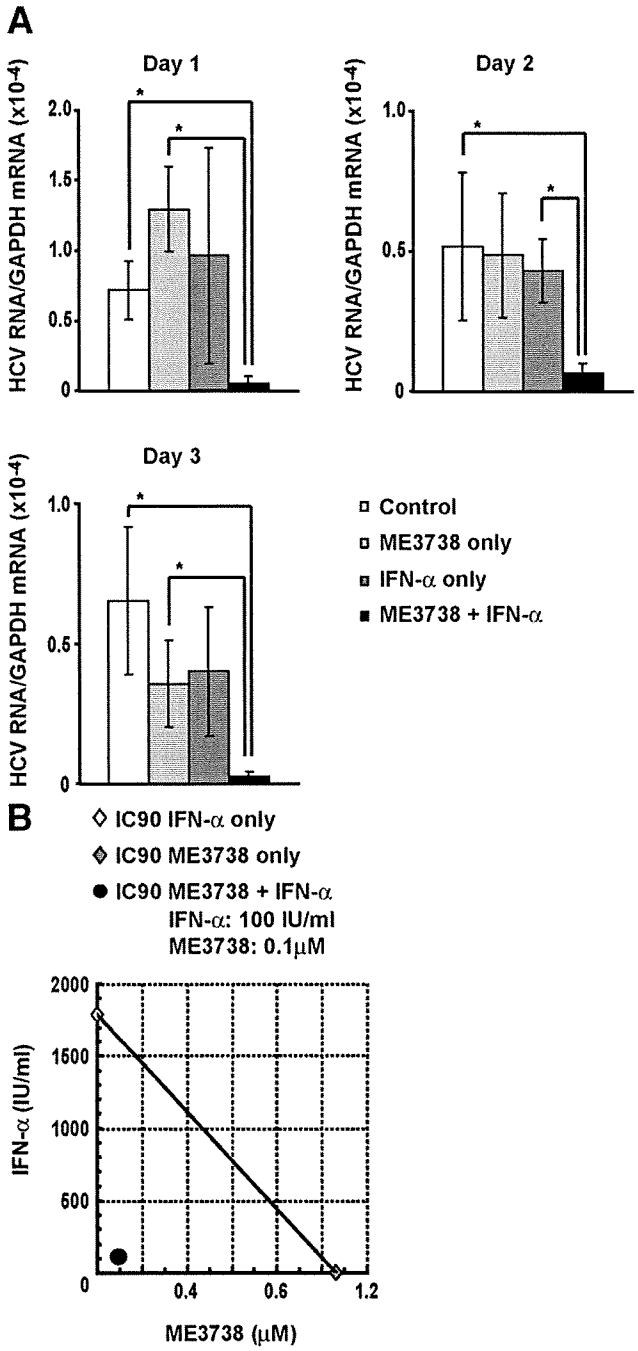


Fig. 6. Synergistic effect of ME3738 with IFN-α on HCV RNA replication. (A) HepG2 cells were infected with Ad-T7pol and then incubated with suboptimal doses of ME3738 (0.1 μM) and IFN-α (100 IU/mL). ME3738 combined with IFN-α significantly reduced HCV replication more than either alone. Data indicate means ± standard error (SE) for three replicates (*$P < 0.05$). (B) Isobole plots of 90% inhibition of HCV RNA replication on day 2 show that IC_{90} of IFN-α alone (white diamond) and of ME3738 alone (gray diamond) were 1792 IU/mL and 1.06 μM, respectively. Solid line indicates additive effect. The black circle in the isobole plot of IC_{90} of ME3738 plus IFN-α indicates synergistic, rather than additive effects of the combination.

Discussion

We showed here that ME3738, a derivative of soyasapogenol, inhibits HCV replication in hepatocyte-derived cell lines. ME3738 enhances autocrine IFN-β production, and this enhancement of IFN-β plays an important role in the anti-HCV effect of ME3738. Combining ME3738 with IFN-α synergistically reduced HCV replication, suggesting that this combination could be an effective therapeutic strategy against HCV.

Huh7 is a highly HCV replication-permissive cell line that has served as the basis of many HCV replication models *in vitro*. One reason why these cells are so permissive for HCV replication is that dsRNA-triggered IFN induction is impaired.[6,23,24] From this perspective, Huh7 might not be suitable for analyzing interactions between IFN and dsRNA, or for screening drugs associated with IFN production. We used an Adeno-T7 delivery plasmid-based HCV replication system because it works in various cell lines, including HepG2 and CV-1. We could monitor HCV replication in HepG2 cell lines using this system, and thus identify ME3738-enhanced IFN-β expression. The anti-HCV effect of ME3738 was obvious in the HepG2 cell line and the expression of both 2′-5′ OAS and MxA mRNA was also significantly and dose-dependently enhanced. Such expression was less remarkable in Huh7, and the anti-HCV effect though evident, was weaker than that in HepG2 cells. Thus, the enhanced IFN-β should be critical for the anti-HCV effect of ME3738. However, ME3738 also has other anti-HCV effects that await characterization since it is a soyasapogenol derivative. Soyasaponin extracted from soybeans (Glycine max L. Merrill) prevents liver damage and hyperlipidemia.[8] Furthermore, HCV replication is associated with cellular lipid droplets,[32] and some lipid metabolic factors are associated with the effect of anti-HCV therapy with IFN.[33] Thus, an indirect effect through lipid metabolism could contribute to the anti-HCV effect of ME3738. Furthermore, soyasaponin has antiviral effects against herpes simplex virus, human cytomegalovirus, influenza virus and human immunodeficiency virus by inhibiting gene and viral protein synthesis.[9,34] The anti-viral effects of ME3738 against HCV could be similar. These issues require further study.

Genotype 1 strains are principally associated with liver diseases worldwide. The most effective antiviral therapy against HCV is presently pegylated IFN combined with ribavirin. However, this strategy eliminates genotype 1 HCV in only about 50% of patients.[2] The remaining 50% requires a more effective therapy with but less side effects to eliminate HCV. ME3738 has anti-HCV effects for genotype 1 HCV.

The present study evaluated the anti-HCV effects of ME3738 alone, as well as in combination with IFN-α. Suboptimal doses of these agents together, remarkably increased anti-HCV activity sooner than either alone.

Since IFN-β induction represents the immediate response of cells to viral infection and precedes the transcription of most IFN-α species, IFN-β enhancement appears to be important for the anti-HCV effect of ME3738. Autocrine IFN-β is recognized as important in viral elimination. Infection with HCV can induce the IFN-β signaling pathway,[35] but HCV has some mechanisms that disrupt this pathway.[27,28] If ME3738 can overcome such disruption, it could increase the antiviral effectiveness against HCV. Moreover, not only hepatocytes, but also immune cells would be affected by autocrine IFN-β,[36] and those influences would help to eliminate HCV. As already demonstrated clinically, the combination of IFN-α and IFN-β enhances antiviral activity against HCV.[37] Therefore, the upregulation of IFN-β by ME3738 could also contribute to reducing HCV replication by combination with IFN-α. Our results demonstrated that ME3738 and IFN combination therapy has synergistic anti-HCV effects, and suggest an additional potential approach toward HCV elimination. Moreover, ME3738 should ameliorate liver damage induced by HCV.[10] Altogether, ME3738 could be a useful anti-HCV strategy, as well as for ameliorating liver damage. These findings support the further evaluation of ME3738 as a promising drug for treating chronic infection with hepatitis C.

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Original Article

Efficacy of splenectomy for hypersplenic patients with advanced hepatocellular carcinoma

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Aim: Chemotherapy for advanced hepatocellular carcinoma (HCC) patients with hypersplenism is generally unsatisfactory, as a lower-dose therapy is usually administered. Splenectomy may represent a better approach to overcoming the complication due to hypersplenism in patients with advanced HCC. This retrospective study was conducted to evaluate whether HCC patients who undergo splenectomy show improved prognosis.

Methods: We examined 34 HCC patients. Twenty-two had thrombocytopenia and/or leucopenia and underwent laparoscopic splenectomy. The completion rate of full-dose drug regimens, the response rate, the toxicity of chemotherapy and the cumulative survival rate were compared between the splenectomy and non-splenectomy groups.

Results: The response rate and the cumulative survival rate in the splenectomy group were significantly better than that in the non-splenectomy group.

Conclusions: Splenectomy is an efficient method for advanced HCC patients with hypersplenism treated by chemotherapy.

Key words: Hepatocellular carcinoma, chemotherapy, splenectomy

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is one of most common malignant tumors. The development of imaging modalities including ultrasonography, computed tomography (CT), and magnetic resonance imaging (MRI) has enabled the early diagnosis of HCC. Thus, curative treatments, such as liver transplantation, hepatic resection, percutaneous ethanol injection, and radiofrequency ablation therapy, are effective in patients with early stage HCC. However, advanced HCC has poor prognosis and a standard therapy has not been established. Advanced HCC is usually associated with liver cirrhosis[1–4] and patients often have hypersplenism due to portal hypertension resulting in anemia, leucopenia, or thrombocytopenia.[5–10] Advanced HCC is widely treated by chemotherapy,[11–13] however as chemotherapy restricts bone marrow function, advanced HCC patients with hypersplenism do not receive a sufficient dose of chemotherapy drugs to be effective. Thus, these patients have been considered to have an even poorer prognosis than advanced HCC patients with splenomegaly who received a more sufficient dose of chemotherapy. Splenectomy may be a better method to overcome the problems of leucopenia and thrombocytopenia in patients with advanced HCC.

We are unable to find in the literature an analysis of the efficacy of splenectomy as an alternative for chemotherapy in cirrhotic patients. This retrospective study aims to evaluate whether HCC patients who undergo splenectomy show improved prognosis.

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PATIENTS AND METHODS

Patients

WE EXAMINED 34 patients (29 males, 5 females; 35–76 years, mean age 59.9 ± 8.7 years) who had been admitted to the Third Department of Internal

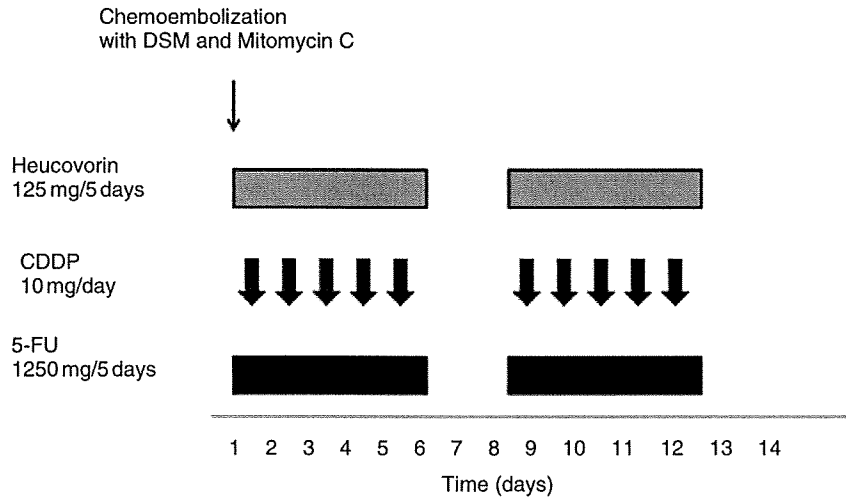

Figure 1 The chemotherapeutic regimen of this study. A schematic presentation of the chemotherapeutic regimen is shown. CDDP, cisplatin; DSM, degradable starch microspheres; 5-FU, 5-fluorouracil.

Medicine, Ehime University Hospital, Japan, between January 2002 and December 2006. The criteria for this study included: (i) a performance status of 0–2; (ii) successful implantation of intra-arterial catheter and drug delivery system; (iii) existence of tumor thrombosis of the portal vein or giant nodule greater than 5 cm in size; and (iv) absence of extra-hepatic metastasis. A diagnosis of HCC was made using imaging analysis, including helical dynamic CT and MRI. The patients with HCC were confirmed to have elevated levels of alpha-fetoprotein (AFP; mean 12241.7 ± 31529.0 ng/mL) and/or des-γ-carboxy-prothrombin (DCP; mean 15428.0 ± 43324.7 mAU/mL). Splenectomy was performed according to the following criteria: (i) thrombocytopenia (platelet count $< 8 \times 10^4$ /mm^3); (ii) leucopenia (white blood cell count $< 2 \times 10^3$/mm^3); or (iii) in the case of consenting splenectomy. Of the 34 patients, 22 with thrombocytopenia and/or leucopenia underwent laparoscopic splenectomy after informed, written consent was obtained (splenectomy group). The splenectomy was performed about 4 weeks before the start of chemotherapy. The remaining 12 patients with advanced HCC who did not consent to a splenectomy were the historical control group (non-splenectomy group). These 12 patients also has thrombocytopenia.

Chemotherapy

A five French heparin-coated catheter was introduced intraluminally to the right femoral artery with a subcutaneously implanted reservoir and was positioned in the proper or common hepatic artery. The gastroduodenal artery and right gastric artery were occluded with steel coils to prevent gastroduodenal injury from the anticancer agents. Patients received regional chemotherapy via the hepatic artery through a subcutaneously implanted port. As shown in Figure 1, one course of chemotherapy consisted of daily administration of cisplatin (CDDP; 10 mg on days 1–5, 8–12) and leucovorin (LV; 25 mg on days 1–5, 8–12) followed by 5-fluorouracil (5-FU; 250 mg on days 1–5, 8–12). In addition, Mitomycin C (8 mg) was emulsified into degradable starch microspheres (DSM), and injected on day 1. This drug regime was repeated weekly for a fortnight and the course of treatment was repeated several times unless the tumor progressed during the therapy.

METHODS

THE CLINICAL PARAMETERS of the patients in each group are shown in Table 1. Laboratory tests were performed before splenectomy in the splenectomy group. All patients had liver cirrhosis. The pathogenesis of liver cirrhosis was hepatitis B virus (HBV) in six patients, hepatitis C virus (HCV) in 27, and unknown etiology in one. Using the Child–Pugh scale, 22 patients were classified as class A cirrohsis and 12 class B. The mean maximum diameter of the HCC nodules was 54.4mm ± 26.0 (20.0–140.0mm). According to the tumor stage defined by the Liver Cancer Study Group of Japan,[14] five patients were in stage 3 and 29 in stage 4.

The efficacy of chemotherapy was assessed by CT during angiography. The response criteria used were those outlined in the Response Evaluation Criteria in Solid Tumors (RECIST)[15–17] guidelines, which propose using only the change in maximal diameter. This led to

Table 1 Characteristics of the patients in the splenectomy and non-splenectomy groups

	Splenectomy group ($n = 22$)	Non-splenectomy group ($n = 12$)	P value
Age (years)	55.8 ± 10.3	62.1 ± 7.0	NS
Gender (M:F)	17:5	12:0	NS
Etiology (HBV:HCV:other)	3:18:1	3:9:0	NS
Child-Pugh grade (A:B)	14:8	8:4	NS
Clinical stage (3:4)	4:18	1:11	NS
Tumor thrombus in main portal branch	8	5	NS
Spleen volume pre-splenectomy (cm^3)	402.8 ± 135.3	361.7 ± 94.4	NS
Platelet count (mm^3)	6.1 ± 1.4	7.3 ± 0.4	0.04
Median white blood cells (mm^3)	3200 (1420–8500)	3900 (2530–5750)	NS
Median tumor diameter (mm)	50.0 (20.5–104.0)	62.0(25.0–78.5)	NS

HBV, hepatitis B virus; HCV hepatitis C virus; NS, not significant.

four categories of response in the target lesion, evaluated as follows: (i) complete response (CR); (ii) total disappearance (TD); (iii) partial response (PR; at least a 30% decrease in the sum of the longest diameter of the target lesion); (iv) progressive disease (PD; at least a 20% increase in the sum of the longest diameter of the target lesion); and (v) stable disease (SD; neither a sufficient shrinkage to qualify for partial response, nor a sufficient increase to qualify for progressive disease). To be assigned a status of CR or PR, changes in tumor measurements needed to be confirmed by assessment performed 4 weeks after the criteria for response were first met. In non-target lesions, response was evaluated as follows: (i) CR (the disappearance of all non-target lesions and normalization of tumor marker level); (ii) incomplete response (SD; the persistence of one or more non-target lesions and/or the maintenance of tumor marker levels above normal limits); (iii) PD (the appearance of one or more new lesions and/or unequivocal progression of existing non-target lesions).

The toxicity of chemotherapy was assessed and graded according to the Common Terminology Criteria for Adverse Events (CTCAE) version 3.0.[18] The completion rate by full dose drugs, the response rate, the toxicity of chemotherapy, and the cumulative survival rate were compared between the splenectomy and non-splenectomy groups. The follow up of albumin, bilirubin, and prothrombin time was performed after splenectomy.

Statistical analysis

Continuous parameters were expressed as medians and ranges (10th and 90th percentiles) or means and standard deviations. Statistical analysis was performed using Student's t-test for paired and unpaired data, Mann–Whitney U-test, χ^2 test, Fisher's exact tests, or Wilcoxon signed-rank test as applicable. The survival curves were plotted using the Kaplan–Meier methodology and were compared using the log-rank test. Multivariate analysis was performed using the Cox proportional hazards regression model. $P < 0.05$ was considered to represent statistical significance.

RESULTS

APART FROM PLATELET count, no significant differences in age, gender, etiology, Child–Pugh grade, clinical stage, tumor diameter, spleen volume before splenectomy or chemotherapy, and white blood cell count (WBC) count were observed between the two groups. The mean platelet counts significantly increased one month after splenectomy ($6.1 \pm 1.4 \times 10^4/mm^3$ vs $18.0 \pm 9.1 \times 10^4/mm^3$, $P < 0.001$). Median WBC counts also increased (3200 [1420–8500] vs. 4600 [2560–7820]). Of the 22 patients in splenectomy group, 20 (90.9%) were able to be treated with the full-dose chemotherapy regimen. Most of those in the non-splenectomy group (8/12, 66.6%) were unable to receive a full therapeutic chemotherapy dose due to thrombocytopenia. In the splenectomy group, two patients were not able to be treated with the full-dose chemotherapy regimen due to infection at the site of the subcutaneously implanted reservoir port.

Table 2 shows the tumor responses. In the splenectomy group, CR was achieved in three patients, PR in 12 patients, yielding a response rate of 68.1%. In the non-splenectomy group, PR was achieved in 2 patients, SD and PD in 10 patients, yielding a response rate of 16.6%. Thus, the response rate of the splenectomy group was significantly better than that of the non-splenectomy group ($P < 0.001$).

Table 2 Comparison of therapeutic response between the splenectomy and non-splenectomy groups

	Splenectomy group ($n = 22$)	Non-splenectomy group ($n = 12$)	P value
Treatment cycle (weeks)	2.9 ± 0.9	2.3 ± 1.0	NS
Full regimen completion (%)	90.9	33.3	<0.001
CR	3	0	
PR	12	2	
SD, PD	7	10	
Response rate (%) 68.1	68.1	16.6	<0.001

CR, complete response; NS, not significant; PD, progressive disease; PR, partial response; SD, stable disease

The cumulative survival rate is shown in Figure 2. The 1-year survival rates for the splenectomy and non-splenectomy groups were 86.3% and 41.6%, respectively, while the 2-year survival rates were 42.5% and 0%, respectively. The cumulative survival rate was significantly better in the splenectomy group than the non-splenectomy group ($P < 0.001$). In all patients, the cause of death was related to the exacerbation of HCC. The most important factors influencing overall survival were splenectomy and the existence of portal thrombus (Table 3). After splenectomy the hazard ratio was 8.89 (CI 2.21–18.82, $P < 0.001$). Post-operative complications consisted of portal thrombosis in only two patients. However the liver function of these patients remained unchanged. Prothrombin time was the only parameter to improve after splenectomy (Table 3).

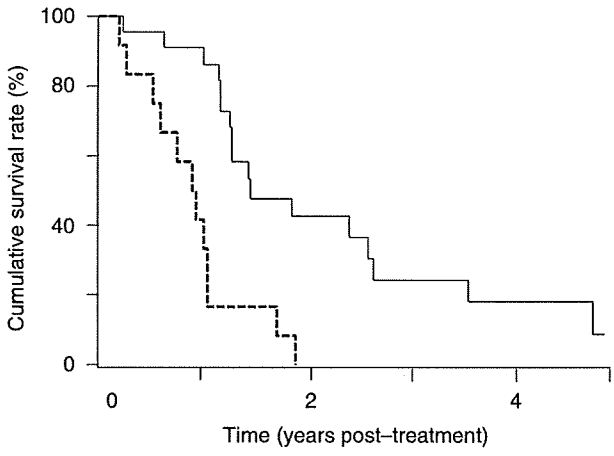

Figure 2 Comparison of cumulative survival rates of the splenectomy (—) and non-splenectomy (- - -) groups. The survival rate of the splenectomy group was significantly higher than that of the non-splenectomy group.

DISCUSSION

THE PROGNOSIS OF advanced HCC has been improved by chemotherapy treatment, especially via hepatic arterial infusion. There are several reports indicating that chemotherapy patients survive more than 3-years post-treatment.[11–13] Hypersplenism, due to portal hypertension, can result in decreased WBC and platelet counts. This is considered a contraindication to chemotherapy, and hypersplenism precludes aggressive chemotherapy. In an attempt to obtain more satisfactory results in the treatment of HCC with hypersplenism, we performed splenectomy prior to chemotherapy. To address the problems caused by hypersplenism, open

Table 3 Multivariable analysis for overall survival

	Hazard ratio (95%CI)	P value
Splenectomy	8.89 (2.21–18.82)	<0.001
Age		
<50	1	
50–59	1.07 (0.21–5.11)	NS
60–69	0.85 (0.15–4.14)	NS
70–	0.47 (0.06–3.52)	NS
Etiology		
HBV	1	
HCV	1.54 (0.47–5.97)	NS
NBNC	1.90 (0.30–4.72)	NS
Child-Pugh		
A	3.44 (0.93–13.05)	NS
B	1	
Tumor diameter		
<50 mm	1	
50 mm–	0.93 (0.30–2.58)	NS
Portal thrombus		
(–)	1	
(+)	2.21 (1.09–4.80)	0.002

HBV, hepatitis B virus; HCV hepatitis C virus; NS, not significant.