

FIG. 3. Interferon-induced expressional induction of the ISGs, P56, PKR, and 25AS in Huh7 cells transfected or infected with wild-type and core mutant JFH1 clones. Two days posttransfection, cells were treated with 50 IU/ml of IFN- α . After 8 h, total cellular RNA was extracted and mRNAs of P56 (A), PKR (B), or 25AS (C) were quantified by real-time RT-PCR analyses. The values are displayed as ratios of IFN-untreated control values. Experiments were repeated three times, and representative results are shown. Asterisks indicate *P* values of less than 0.05 compared to results for the wild type. (D) Western blotting. Expression of total and phosphorylated STAT1 and STAT2 proteins in cells transfected with the wild type and core mutant HCV clones. (E) Western blotting. Expression of total and phosphorylated STAT1 and STAT2 proteins in cells infected with the wild type and core mutant HCV clones. Densitometries for pSTAT1 and pSTAT2 were performed, and results are shown as percentage of results for HCV-negative samples.

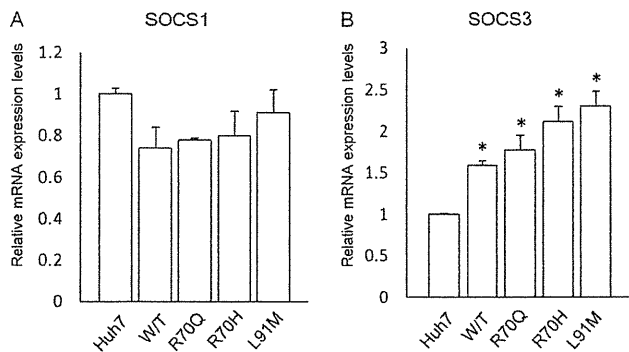


FIG. 4. Effects of core mutant HCV on SOCS1 and SOCS3 expression in Huh7 cells. Expression levels of SOCS1 (A) or SOCS3 (B) in Huh7 cells transfected with the wild type or the core mutant JFH1. Three days posttransfection, total cellular RNA was isolated and the mRNA was quantified by real-time RT-PCR analyses. The experiments were repeated three times, and representative results are shown. The values are displayed as values relative to beta-actin levels. Each experiment was repeated three times, and the representative results are shown. Asterisks indicate *P* values of less than 0.05 compared to results for the wild type.

mRNA between cells transfected with the wild type and the core mutant clones. In contrast, the SOCS3 mRNA expression level was significantly higher in core mutant-transfected cells than in wild-type-transfected cells (Fig. 4A and B). It is known that SOCS3 is induced principally by phosphorylated STAT3 (pSTAT3) (18) and that interleukin-6 (IL-6) is a strong inducer of pSTAT3 via receptor-mediated Janus kinase activation in the liver (41, 51). On that basis, we investigated whether overexpression of SOCS3 is associated with increased pSTAT3 and with overproduction of IL-6. The pSTAT3 level was significantly higher in core mutant-transfected cells than in JFH1-transfected cells and naive Huh7 cells (Fig. 5A). Moreover, cellular IL-6 mRNA expression was significantly higher in core mutant-transfected cells than in wild-type-transfected cells (Fig. 5B). These findings suggested that upregulation of cellular SOCS3 is associated with the resistance to IFN of the core 70/91 mutant HCV clones and that this effect is mediated partly by overproduction of IL-6.

UPRs are enhanced in core mutant-transfected cells. We have reported that HCV causes direct cytopathic effects on host cells and that these effects are mediated by HCV-induced unfolded protein responses (UPRs) (48). Therefore, we detected the expression of UPR-related proteins, GRP78 and CHOP, in cells expressing wild-type HCV and the core 70/91 mutants. As shown in Fig. 6, HCV-transfected cells showed higher expression levels of GRP78 and CHOP than untransfected cells. Furthermore, cells transfected with HCV core 70/91 mutant clones expressed larger amounts of GRP78 and CHOP than the wild-type-transfected cells. Because IL-6 is principally expressed following UPR induction (Fig. 5B), these data indicate that HCV-induced UPR may be involved in the IFN resistance of core mutant clones.

DISCUSSION

In this study, we used a virus cell culture system to investigate the characteristics of R70Q, R70H, and L91M HCV core

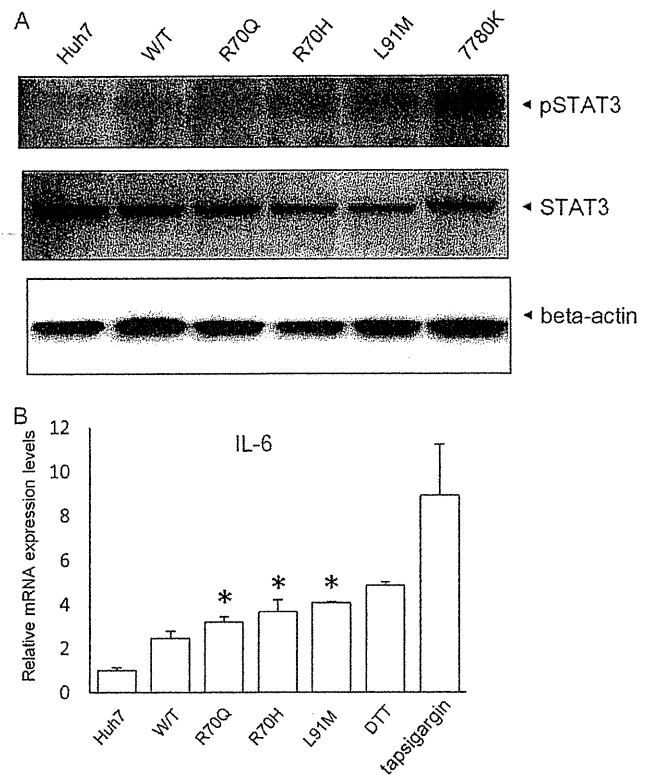


FIG. 5. Expression of phosphorylated STAT3 and IL-6 in cells transfected with the wild type and core mutant HCV-JFH1 clones. (A) Western blotting. Expression of total and phosphorylated STAT3 and beta-actin proteins in cells transfected with the wild type or core mutant HCV clones. (B) Two days posttransfection, total cellular RNA was extracted and mRNAs of IL-6 were quantified by real-time RT-PCR analyses. The values are displayed as the ratio of values of the HCV-untreated control. Asterisks indicate *P* values of less than 0.05 compared to results for the wild type.

mutant viruses, which were clinically resistant to peg-IFN-plus-RBV treatment, and found that these core mutant clones showed resistance to IFN *in vitro*, consistent with the clinical findings (Fig. 1). These differences in the IFN sensitivity of the core mutant clones led us to conduct a series of experiments to investigate the molecular mechanisms of IFN-related response pathways. We found that IFN- α receptor-mediated signaling was attenuated in wild-type HCV-infected and core mutant-infected cells compared to that in uninfected cells and that the suppression of IFN signaling was more potent for core mutant clones than for the wild type. The differences in the interferon-mediated antiviral effects were demonstrated further by the difference in the induction rates of IFN-inducible P56, PKR, and 25AS mRNAs (Fig. 3A, B, and C) and IFN-induced phosphorylation of STAT1 and STAT2 (Fig. 3D and E). Furthermore, the expression levels of an interferon signal attenuator, SOCS3, were significantly higher in core mutant-transfected cells than in wild-type-transfected cells. Moreover, cellular expression of IL-6, which induces SOCS3 expression through phosphorylation of STAT3 (18, 41), was significantly higher in the core mutant-transfected cells than in wild-type-transfected cells (Fig. 5A). Taking all these things together, it is suggested strongly that the IFN resistance of core mutant clones is due to

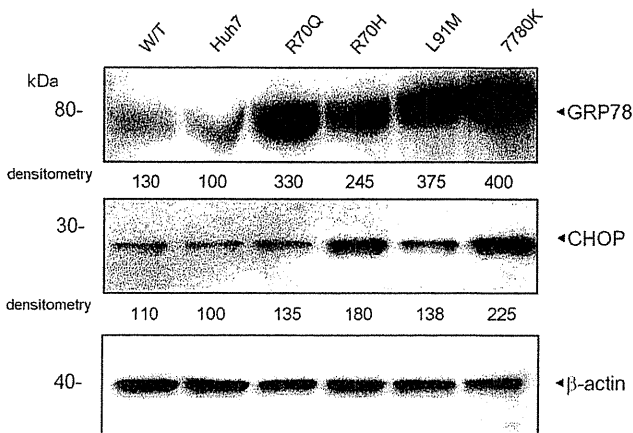


FIG. 6. Expression of GRP78 and CHOP UPR genes in cells transfected with the wild type and core mutant HCV-JFH1 clones. Western blotting was performed to assess UPR following transfection with HCV core mutants. Ten micrograms of harvested cell lysates were subjected to Western blotting using anti-GRP78 and anti-CHOP antibodies. Densitometries for GRP78 and CHOP were performed, and results are shown as percentages of results for uninfected cells.

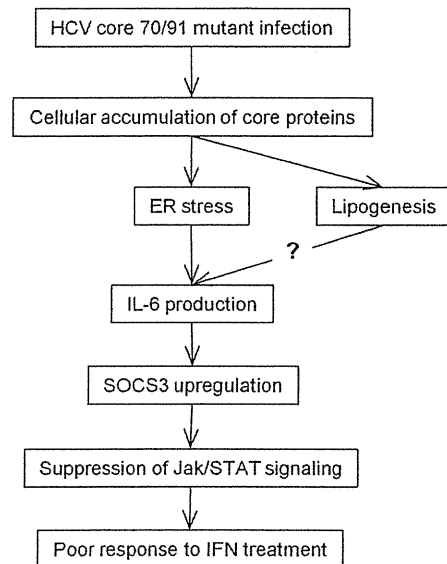


FIG. 7. Schematic diagram of signaling pathway involved in HCV core mutant infection and IFN resistance.

SOCS3-mediated attenuation of IFN responses and that, more importantly, upregulation of cellular IL-6 is attributable to emergence of IFN resistance (Fig. 7).

Miyazaki et al. demonstrated that core protein, which is localized in LD-associated membrane, recruits HCV nonstructural (NS) proteins and replication complexes to LD and that this recruitment is critical for producing infectious viruses (35). Furthermore, Masaki et al. reported that the NS5A protein interacts with core at its C-terminal serine cluster and this NS5A-core interaction is crucial for the production of virus particle (32). In this study, there was no difference between the core mutants and the wild-type virus in terms of the pattern of colocalization of core protein with LDs and also the ER membrane (see the figure in the supplemental material). These results suggest that the core amino acid substitutions at positions 70 and 91 do not alter the characteristics of the core protein in terms of subcellular localization. Murray et al. conducted a comprehensive alanine substitution scan of the core protein to search for domains that are essential for virion production. They showed that substitutions of amino acids 70 and 91 spared but slightly decreased the capacity for virus particle production (37), which is consistent with our present results. Those mutations may cause accumulation of virus and core protein in the LDs and ER membrane and may elicit UPRs and IFN resistance.

Type I IFNs and their responsive ISGs are the principal mediators of host defense against virus infections, including HCV (10, 26, 44). Upon binding of IFNs to their receptors, IFNAR1 and IFNAR2, Janus kinases (Jak)1 and 2 phosphorylate STAT1 and STAT2 to form ISGF-3, which translocates to the nucleus and activates transcription of ISGs (46, 54, 55). Members of the SOCS family are potent inhibitors of type I and type III IFN-induced activation of the Jak-STAT pathway and subsequent expression of ISGs (58). HCV, on the other hand, counteracts such IFN-mediated antiviral pathways through its interaction with various steps of IFN signaling. The

HCV NS5A and E2 proteins interfere with the action of IFN by inhibiting the activity of PKR (20, 56). NS5A also induces expression of IL-8 and attenuates expression of ISGs (40).

HCV core protein has been reported to interfere with the antiviral actions of IFN. Core protein binds the STAT1-SH domain (29) and destabilizes STAT1 (28) to block IFN signaling. Blindenbacher et al. (8) showed that STAT signaling was strongly inhibited in the hepatocytes of HCV core transgenic mice. Bode et al. showed that HCV core protein induced SOCS3 expression and inhibited tyrosine phosphorylation of STAT1 in HepG2 cells (9). In this study, we used full-length HCV cell culture and found that SOCS3 expression is upregulated at different rates, depending on the genetic sequences of HCV strains, and that these differences in SOCS3 expression are associated with sensitivity to IFN. These results indicate that the IFN resistance of HCV-infected cells is mediated by overexpression of SOCS3, which may be upregulated by HCV proteins, as previously reported (9, 27). Only one amino acid difference, R70Q, R70H, or L91M, might have affected cellular responses to interferon.

IL-6 is the principal activator of STAT3 in hepatocytes (18, 41). It has been reported that plasma IL-6 levels are elevated in CHC patients (30). Basu et al. have conducted DNA microarray analyses in HCV core-expressing cells and demonstrated that genes including those encoding IL-6 and STAT3 were upregulated by core protein (7). Consistent with these findings, we found that cellular IL-6 expression levels were elevated in HCV-transfected cells in the order (from lowest to highest levels) untransfected, wild type, and then core mutants, which correlated well with SOCS3 expression (Fig. 4B) and with cellular responses to IFN (Fig. 1B and C). The inducers of IL-6 remain to be clarified. IL-6 is secreted in response to cellular steatosis and insulin resistance (45). Hepatic steatosis is found in 70% of CHC patients (57) and those with obesity; steatosis or insulin resistance is refractory to IFN treatment (43). Such patients show higher levels of hepatic SOCS3 ex-

pression than those without obesity or insulin resistance (34, 61). We reported previously that a series of genes involved in fatty acid and cholesterol synthesis are upregulated in HCV replicon-expressing and HCV-JFH1-infected cells and increased cellular LDs (39). Such lipogenic cellular processes may be the cause of the upregulated expression of IL-6. Alternatively, UPRs may produce IL-6. Chen et al. have reported that UPRs are coupled with TNF- α and IL-6 production in human macrophages (11). In this study, transfection of Huh7 cells by HCV induced the expression of UPR genes, and their expression levels were significantly higher in mutant core protein-transfected cells than in wild type-transfected cells (Fig. 6).

The differences in ISG expression levels between the HCV wild type and core mutants were significant but small (Fig. 3A, B, and C). As shown in Fig. 3D, and E and 4B, the interclone differences in pSTAT and SOCS3 were significant but relatively small, which may explain the small differences in ISG levels. Similarly, the clinical difference in interferon treatment outcomes between core 70/91 mutants and wild types are significant but are around the sustained viral clearance rates of 32.4% versus 53.5% in core 70 or 91 mutants and wild types, respectively (19), which might be consistent with our present results.

In clinical settings, IFN resistance of the core amino acid 70/91 mutants has been reported for genotype 1b strains (5). At present, there is no report that these mutations are associated with IFN treatment responses to other genotypes, including genotype 2a, which we used in this study. Because HCV strains other than genotypes 1 and 4 are generally sensitive to IFN, the core 70/91 mutations might not affect final treatment outcomes. We have conducted preliminary experiments using genotype 1b infectious clones with low levels of replication and found that these mutations did not significantly affect sensitivity to IFN in culture. It may be necessary to investigate IFN sensitivity when efficient cell culture systems have been developed for HCV genotype 1.

In addition to the poor virological responses of HCV core amino acid 70/91 mutants to peg-IFN-plus-RBV treatment (4, 6, 12), patients infected with the core mutants showed increased incidence of hepatocellular malignancies (2, 15, 49). It has been reported that the HCV core R70 but not L91 mutant frequently causes steatosis and increased hepatic oxidative stress (52). It is possible that core 70/91 mutations not only induce IFN resistance but also may cause other pathophysiological conditions, such as carcinogenesis and disorders of lipid metabolism.

In conclusion, our study demonstrates that the IFN resistance of HCV core mutants may be, for the most part, determined by cellular expression levels of SOCS3 and IL-6. Therapeutic targeting of IL-6 potentially may be a key to targeting IFN resistance and improving antiviral chemotherapeutics against HCV.

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Association of IL28B Variants With Response to Pegylated-Interferon Alpha Plus Ribavirin Combination Therapy Reveals Intersubgenotypic Differences Between Genotypes 2a and 2b

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Genetic polymorphisms of the interleukin 28B (IL28B) locus are associated closely with outcomes of pegylated-interferon (PEG-IFN) plus ribavirin (RBV) combination therapy. The aim of this study was to investigate the relationship between IL28B polymorphism and responses to therapy in patients infected with genotype 2. One hundred twenty-nine chronic hepatitis C patients infected with genotype 2, 77 patients with genotype 2a and 52 patients with genotype 2b, were analyzed. Clinical and laboratory parameters, including genetic variation near the IL28B gene (rs8099917), were assessed. Drug adherence was monitored in each patient. Univariate and multivariate statistical analyses of these parameters and clinical responses were carried out. Univariate analyses showed that a sustained virological response was correlated significantly with IL28B polymorphism, as well as age, white blood cell and neutrophil counts, adherence to RBV, and rapid virological response. Subgroup analysis revealed that patients infected with genotype 2b achieved significantly lower rapid virological response rates than those with genotype 2a. Patients with the IL28B-major allele showed higher virus clearance rates at each time point

than those with the IL28B-minor allele, and the differences were more profound in patients infected with genotype 2b than those with genotype 2a. Furthermore, both rapid and sustained virological responses were associated significantly with IL28B alleles in patients with genotype

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; IFN, interferon; PEG-IFN, pegylated-interferon; RBV, ribavirin; IL28B, interleukin 28B; SNPs, single nucleotide polymorphisms; BMI, body mass index; ALT, alanine transaminase; ISDR, the interferon sensitivity determining region; ITPA, inosine triphosphatase

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2b. IL28B polymorphism was predictive of PEG-IFN plus RBV combination treatment outcomes in patients infected with genotype 2 and, especially, with genotype 2b. In conclusion, IL-28B polymorphism affects responses to PEG-IFN-based treatment in difficult-to-treat HCV patients. *J. Med. Virol.* 2011 Wiley-Liss, Inc.

KEY WORDS: hepatitis C virus (HCV); chronic hepatitis C; genotype 2; PEG-IFN plus RBV therapy; combination therapy; IL28B; interferon-13

INTRODUCTION

Hepatitis C virus (HCV) infects around 170 million people worldwide and is characterized by a high probability of developing chronic inflammation and fibrosis of the liver, leading to end-stage liver failure and hepatocellular carcinoma (HCC) [Alter, 1997; Sakamoto and Watanabe, 2009]. Since the first report in 1986, type I interferons have been the mainstay of HCV therapy [Hoofnagle, 1994]. Current standards of care consist of a combination of ribavirin (RBV) plus pegylated interferon (PEG-IFN)-alpha for 48 weeks for infection with genotypes 1 and 4, and for 24 weeks for the other genotypes [Zeuzem et al., 2000; Fried et al., 2002]. Although this treatment improved substantially sustained virological response rates, it may result also in serious adverse effects and a considerable proportion of patients require early discontinuation of treatment. Patients of African origin have even poorer treatment outcomes [Rosen and Gretch, 1999]. Given this situation, a precise assessment of the likely treatment outcomes before the initiation of treatment may improve substantially the quality of antiviral treatment.

Recently, several studies have reported that genetic polymorphisms of the IL28B locus, which encodes interferon-13 (interleukin 28B), are associated with response to interferon-based treatment of chronic HCV infections with genotype 1 [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009] and also spontaneous clearance of HCV [Thomas et al., 2009].

While chronic HCV infections with genotype 2 are associated with good treatment outcome, there are some refractory cases among patients infected with genotype 2, similar to genotype 1. The aims of this study were to analyze retrospectively clinical and virological factors associated with treatment response in patients with chronic HCV infection with genotype 2 who were treated with PEG-IFN plus RBV combination therapy and to clarify the relationship between IL28B polymorphism and the response to combination therapy.

PATIENTS AND METHODS

The authors analyzed retrospectively 129 patients with chronic HCV infection with genotype 2 who

received combination therapy with PEG-IFN plus RBV between December 2004 and December 2009 at 10 multicenter hospitals (liver units with hepatologists) throughout Japan. All patients had chronic active hepatitis confirmed histologically or clinically and were positive for anti-HCV antibodies and serum HCV RNA by quantitative or qualitative assays. Patients with a positive test for serum hepatitis B surface antigen, coinfection with other HCV genotypes, coinfection with human immunodeficiency virus, other causes of hepatocellular injury (such as alcoholism, autoimmune hepatitis, primary biliary cirrhosis, or a history of treatment with hepatotoxic drugs), and a need for hemodialysis were excluded.

Study Design

Each patient was treated with combination therapy with PEG-IFN-a2b (Peg-Intron, Schering-Plough Nordic Biotech, Stockholm, Sweden, at a dose of 1.2–1.5 mg/kg subcutaneously once a week) or PEG-IFN-a2a (Pegasys; Roche, Basel, Switzerland, at a dose of 180 mg subcutaneously once a week) plus RBV (Rebetol, Schering-Plough Nordic Biotech or Copegus; Roche) 600–1,000 mg daily depending on the body weight (b.w.) (b.w. <60 kg: 600 mg po daily; b.w. 60–80 kg: 800 mg po daily; b.w. >80 kg: 1,000 mg po daily; in two divided doses). The duration of the combination therapy was set at a standard 24 weeks, but treatment reduction or discontinuation was permitted by doctor's decision. The rates of PEG-IFN and RBV administration achieved were calculated as percentages of actual total dose administered of a standard total dose of 24 weeks, according to body weight before therapy. During treatment, patients were assessed as outpatients at weeks 2, 4, 6, 8, and then every 4 weeks for the duration of treatment and at every 4 weeks after the end of treatment. Biochemical and hematological testing was carried out in a central laboratory. Serum HCV RNA was measured before treatment, during treatment at 4 weekly intervals, and after therapy at 4 weekly intervals for 24 weeks, by quantitative or qualitative assays.

Patient Evaluation

The following factors were analyzed to determine whether they were related to the efficacy of combination therapy: age, gender, body mass index (BMI), previous IFN therapy, grade of inflammation and stage of fibrosis on liver biopsy, pretreatment biochemical parameters, such as white blood cells, neutrophils, hemoglobin, platelet count, alanine transaminase (ALT) level, serum HCV RNA level (log IU/ml), and single nucleotide polymorphism (SNPs) in the IL28B locus (rs8099917). Liver biopsy specimens were evaluated blindly, to determine the grade of inflammation and stage of fibrosis, by an independent interpreter who was not aware of the clinical data. Activity of inflammation was graded on a scale of 0–3: A0 shows no activity, A1 shows mild activity, A2 shows moderate activity and A3 shows severe activity. Fibrosis was staged on a scale of 0–4:

F0 shows no fibrosis, F1 shows moderate fibrosis, F2 shows moderate fibrosis with few septa, F3 shows severe fibrosis with numerous septa without cirrhosis and F4 shows cirrhosis.

Informed written consent was obtained from each patient who participated in the study. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and to the relevant ethical guidelines as reflected in a priori approval by the ethics committees of all the participating universities and hospitals.

SNP Genotyping

Human genomic DNA was extracted from whole blood of each patient. Genetic polymorphism of IL28B was determined by DigiTag2 assay by typing one tag SNP located within the IL28B locus, rs8099917 (22). Heterozygotes (T/G) or homozygotes (G/G) of the minor allele (G) were defined as having the IL28B minor allele, whereas homozygotes for the major allele (T/T) were defined as having the IL28B major allele.

Outcomes

The primary end point was a sustained biochemical and virological response. A sustained virological response was defined as serum HCV RNA undetectable at 24 weeks after the end of treatment. Secondary end points were a rapid virological response (HCV RNA undetectable in serum at week 4) and end-of-treatment virological response. In addition, tolerability (adverse events) and drug adherence were recorded and factors potentially associated with virological response explored.

Statistical Analysis

SPSS software package (SPSS 18J, SPSS, Chicago, IL) was used for statistical analysis. Discrete variables were evaluated by Fisher's exact probability test and distributions of continuous variables were analyzed by the Mann-Whitney U-test. Independent factors possibly affecting response to combination therapy were examined by stepwise multiple logistic-regression analysis. All P-values were calculated by two-tailed tests, and those of less than 0.05 were considered statistically significant.

RESULTS

Clinical Characteristics and Response to Therapy

The clinical characteristics and response rates to therapy of 129 patients are summarized in Tables I and II. Sixty-eight patients achieved a rapid virological response, whereas 44 patients remained HCV-RNA positive at week 4. Treatment reduction or cessation was permitted also to avoid side effects, and one patient stopped treatment at week 12 because he was

TABLE I. Baseline Characteristics of Participating Patients Infected With HCV Genotype 2

Total number	129
Genotype (2a/2b)	77/52
IL28B SNPs (rs8099917)	
TT/TG/GG	100/28/1
Age (years) ^a	64 (20–73)
Gender (male/female)	64/65
Body mass index (kg/m ²) ^a (N 80)	23.7 (16.9–33.5)
Previous interferon therapy (no/yes)	102/21 (unknown 6)
Histology at biopsy (N 96)	
Grade of inflammation	
A0/1/2/3	10/53/29/4
Stage of fibrosis	
F0/1/2/3	7/59/19/11
White blood cells (/ml) ^b (N 94)	5,115 1,630
Neutrophils (/ml) ^b (N 94)	2,765 1,131
Hemoglobin (g/dl) ^b (N 95)	14.2 1.3
Platelet count (10 ³ /ml) ^b (N 98)	187 95
ALT (IU/L) ^b (N 95)	82 78
Serum HCV-RNA level (log(IU/ml)) ^{a,c}	6.2 (3.6–7.4)
Treatment duration (>16, ≥24)	19/110

SNPs, single nucleotide polymorphisms; ALT, alanine transaminase.

^aData are shown as median (range) values.

^bData are expressed as mean SD.

^cData are shown as log(IU/ml).

anticipated to be a non-responder. On an intention-to-treat analysis, serum HCV-RNA levels were negative at the end of treatment in 125 of the 129 patients (97%) treated and, among them, 98 (76%) achieved a sustained virological response. The rapid virological response rate of patients infected with genotype 2b was lower significantly than that of patients infected with genotype 2a (P 0.036) (Table II). The sustained virological response rate decreased with RBV drug discontinuation and dose reduction (84% and 66% with ≥80% and <80% of RBV dose, P 0.021, Table III). Adherences to PEG-IFN did not influence a sustained virological response or end of treatment response significantly, while RBV adherence was associated significantly with a sustained virological response (Table III).

Factors Associated With a Sustained Virological Response

Next the host clinical and viral factors associated with a sustained virological response were analyzed. Univariate statistical analysis showed that six parameters were associated significantly with the sustained virological response rates, including age, white blood cells, neutrophils, adherence to RBV, rapid virological response and an IL28B SNP (rs8099917) (Table IV). There was no significant association of sustained virological response with gender, previous interferon therapy, stage of fibrosis, pretreatment HCV titer or adherence to PEG-IFN. Further multivariate analyses were conducted using significant factors identified by the univariate analysis (Table V). The multiple logistic-regression analysis showed that only a rapid virological response was associated with a sustained virological response (OR 0.170, P 0.019).

TABLE II. Response Rates to Therapy

Character	Number/total number (%)		
Overall			
RVR	68/112 (61)		
ETR	125/129 (97)		
SVR	98/129 (76)		
Genotype	2a	2b	P-value
RVR	46/67 (69)	22/45 (49)	0.036
ETR	74/77 (96)	51/52 (98)	NS
SVR	56/77 (73)	42/52 (81)	NS

RVR, rapid virological response; ETR, end of treatment response; SVR, sustained virological response. Bold indicated P-value of less than 0.05.

TABLE III. Response Rates to Treatment According to Drug Adherence

	≥80%	<80%	P-value
PEG-IFN adherence			
ETR	94/96 (98)	31/33 (94)	NS
SVR	75/96 (78)	23/33 (70)	NS
RBV adherence			
ETR	72/73 (99)	53/56 (95)	NS
SVR	61/73 (84)	37/56 (66)	0.021

ETR, end of treatment response; SVR, sustained virological response; PEG-IFN, pegylated interferon; RBV, ribavirin. The rates of PEG-IFN and RBV administration achieved were calculated as percentages of actual total dose administered of a standard total dose of 24 weeks, according to body weight before therapy. Bold indicated P-value of less than 0.05.

Comparison of Sustained Virological Response Rates According to IL28B SNPs

The PEG-IFN plus RBV treatment efficacy was compared after dividing the study subjects into two groups based on IL28B alleles (Table VI). Patients homozygous for the IL28B major allele (TT allele) achieved significantly higher rapid and sustained virological response

rates than those heterozygous or homozygous for the IL28B minor allele (TG/GG alleles) ($P < 0.05$). In addition, responses to PEG-IFN plus RBV treatment were analyzed after dividing the study subjects into those with genotype 2a and with genotype 2b. The rapid and sustained virological response rates tended to be higher in patients homozygous for the IL28B major allele than those heterozygous or homozygous for the

TABLE IV. Clinical and Virological Characteristics of Patients Based on Therapeutic Response

	SVR (n = 98)	Non-SVR (n = 31)	P-value
Genotype (2a/2b)	56/42		21/10
IL28B SNPs (rs8099917)			
TT/TG GG	81/17	19/12	0.024
Age (years) ^a	56 (20–73)	61 (40–72)	0.002
Gender (male/female)	51/47	13/18	NS
Body mass index (kg/m ²) ^a	22.8 (16.9–33.5)	24.1 (20.3–27.6)	NS
Previous Interferon therapy (no/yes)	80/14	22/7	NS
Grade of inflammation (A0-1/2-3)	46/28	15/7	NS
Stage of fibrosis (F0-2/3-4)	64/10	21/1	NS
White blood cells (/ml) ^b	5,318 1,617	4,489 1,540	0.032
Neutrophils (/ml) ^b	2,913 1,139	2,278 983	0.021
Hemoglobin (g/dl) ^b	14.2 1.4	14.1 1.1	NS
Platelet count (10 ³ /ml) ^b	193 105	171 54	NS
ALT (IU/ml) ^b	79 73	94 92	NS
Pretreatment Serum HCV-RNA level (log(IU/ml)) ^{a,c}	6.1 (3.6–7.4)	6.3 (4.0–6.7)	NS
PEG-IFN adherence (≥80%/<80%)	75/23	21/10	NS
RBV adherence (≥80%/<80%)	61/37	12/19	0.024
RVR/non-RVR	57/24	11/20	0.001

SNPs, single nucleotide polymorphisms; ALT, alanine transaminase; RVR, rapid virological response.

^aData are shown as median (range) values.

^bData are expressed as mean ± SD.

^cData are shown as log (IU/ml).

Bold indicated P-value of less than 0.05.

TABLE V. Multivariate Analysis for the Clinical and Virological Factors Related to Sustained Response With Peg-IFN Plus RBV Therapy in 63 Patients

Factor	Category	Odds ratio (95% CI)	P-value
Regression analysis			
RVR	RVR	1	0.019
	Non-RVR	0.170 (0.039–0.744)	
RBV adherence	≥80%	1	0.061
	<80%	0.250 (0.059–1.064)	
IL28B SNPs (rs8099917)	TT	1	0.104
	TG GG	0.252 (0.048–1.330)	
Age		1.087 (0.976–1.211)	0.128
Neutrophils		0.999 (0.997–1.001)	0.209
White blood cells		1.000 (0.999–1.002)	0.504

CI, confidence interval; SNPs, single nucleotide polymorphisms; RVR, rapid virological response, RBV, ribavirin.
Bold indicated P-value of less than 0.05.

IL28B minor allele infected with both genotype 2a and 2b, and these differences were more profound in patients infected with genotype 2b than with genotype 2a. The rapid and sustained virological response rates of patients with the major IL28B allele were higher significantly than those of patients with the minor IL28B allele infected only with genotype 2b (rapid virological response: 58% and 0% with IL28B major and hetero/minor, P 0.002, sustained virological response: 88% and 44% with IL28B major and hetero/minor, P 0.009).

Although the rapid virological response rate of patients infected with genotype 2b was lower significantly than that of patients infected with genotype 2a, the sustained virological response rate was higher in patients infected with genotype 2b than with genotype 2a (Table II). In order to investigate that discrepancy, sustained virological response rates in patients with or without rapid virological response were analyzed according to IL28B SNPs. In patients infected with genotype 2b and a non-rapid virological response, the sustained virological response rates differed significantly between IL28B major and hetero/minor groups (sustained virological response with non-rapid virological response: 75% and 29% with IL28B major and hetero/minor, P 0.044), and no one achieved a rapid

virological response among the patients infected with genotype 2b and with the IL28B hetero/minor allele. In patients infected with genotype 2a, on the contrary, there was no significant correlation of rapid and sustained virological response rates between IL28B SNPs (sustained virological response with rapid virological response: 78% and 70% with IL28B major and hetero/minor, P 0.630, sustained virological response with non-rapid virological response: 57% and 43% with IL28B major and hetero/minor, P 0.552).

Next, changes in virological response rates over time were investigated in patients treated with PEG-IFN plus RBV and the time course was analyzed after separating the patients infected with genotype 2a and 2b (Fig. 1). Patients with IL28B-TG and -GG showed significantly lower rates of rapid and sustained virological response, compared to patients with IL28B-TT, and greater differences were observed according to IL28B SNPs among patients infected with genotype 2b than with 2a.

Side Effects

Side effects leading to Peg-IFN plus RBV discontinuation occurred in eight patients (6.2%) and discontinuation of RBV alone occurred in four patients (3.1%).

TABLE VI. Rapid and Sustained Virological Response Rates to Treatment According to IL28B SNPs

Character	IL28B major	IL28B hetero/minor	P-value
Number/total number (%)			
Overall			
RVR	58/88 (66)	10/24 (42)	0.031
SVR	81/100 (81)	17/29 (59)	0.013
Genotype 2a			
RVR	36/50 (72)	10/17 (59)	NS
SVR	43/57 (75)	13/20 (65)	NS
Genotype 2b			
RVR	22/38 (58)	0/7 (0)	0.002
SVR	38/43 (88)	4/9 (44)	0.009

RVR, rapid virological response; ETR, end of treatment response; SVR, sustained virological response.

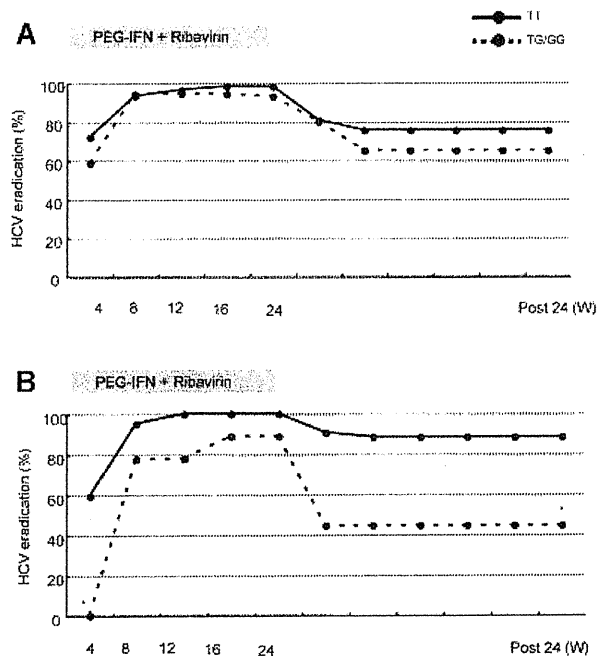


Fig. 1. Changes over time in virological response rates were confirmed in patients treated with PEG-IFN plus RBV, and the time courses were analyzed after separating the patients infected with genotypes 2a and 2b. Patients with the IL28B major (TT allele) are indicated in the figure by a continuous line and those with IL28B hetero or minor (TG or GG), by a dotted line. IL28B-TG and -GG patients showed significantly lower rates of rapid and sustained virological response, compared to IL28B-TT patients. P-values were two-tailed and those of less than 0.05 were considered to be statistically significant. $P < 0.01$.

Among the eight patients who withdrew from both drugs, four, including one who stopped at week 7, had achieved a sustained virological response. Among four patients who withdrew from RBV alone, three had achieved a sustained virological response. The events leading to drug withdrawal were HCC treatment (n 2), general fatigue (n 2), retinopathy, neuro-psychiatric event, severe dermatological symptoms suggestive of the drug-induced hypersensitivity syndrome, and arrhythmia.

DISCUSSION

Recent studies suggest that genetic variations in IL28B are strongly associated with response to therapy of chronic HCV infection with genotype 1 [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009] and with spontaneous HCV clearance [Thomas et al., 2009]. In this study, univariate analyses showed that the sustained virological response was correlated significantly with IL28B polymorphism (rs8099917) as well as age, adherence to RBV and rapid virological response, and multiple logistic-regression analysis showed that only a rapid virological response was associated with a sustained virological response in all patients infected with genotype 2 (Table V). Although the IL28B

polymorphisms are not so useful for predicting the clinical outcomes of PEG-IFN plus RBV combination therapy among patients with genotype 2, compared to genotype 1, IL28B polymorphism was predictive of PEG-IFN plus RBV treatment outcomes among patients with genotype 2 and, more remarkably, among patients with genotype 2b in this study. Indeed, both rapid and sustained virological response rates according to the rs8099917 genotypes were different significantly in patients with genotype 2b but not in patients with genotype 2a. Furthermore, in the plot of virological response (Fig. 1), a stronger effect of the IL28B allele was observed in patients with genotype 2b than with genotype 2a.

It has been reported that there was no significant association between genetic variation in IL28B and response to therapy of HCV patients infected with genotype 2 or 3, indicating that the prognostic value of the risk allele for treatment response might be limited to individuals with difficult-to-treat HCV genotypes [Rauch et al., 2010]. This report lacks details of the distribution of the various genotypes. The present study agrees with a more recent report that the IL28B polymorphism was associated with a sustained virological response in patients with chronic HCV infection with genotype 2 or 3 who did not achieve a rapid virological response [Mangia et al., 2010]. In Japan, the percentage of HCV infection with genotype 1b is 70%, genotype 2a is 20% and genotype 2b is 10%, whilst other genotypes are observed only rarely. In this study, the association of IL28B polymorphism with response to therapy was analyzed in more detail, considering the subtypes 2a and 2b, and IL28B polymorphism (rs8099917) found to be linked more closely to the virological response of patients infected with genotype 2b than those with genotype 2a. A recent *in vitro* study, which constructed several chimeric virus clones between HCV-2b and HCV-JFH1 (2a), also supported subgenotypic differences between genotype 2a and 2b [Suda et al., 2010]. The authors speculated that the prognostic value of the risk allele for treatment response might be more pronounced in individuals with difficult-to-treat HCV subgenotypes, such as patients infected with genotype 2b, compared with 2a. In addition, the prevalence of the IL28B minor allele is much higher in Caucasians and African Americans than in eastern Asian populations [Thomas et al., 2009], which suggest that the effects of IL28B polymorphism could be more pronounced in non-Asian populations. In the present results, however, the sustained virological response rate of patients infected with genotype 2b was higher than that of patients with genotype 2a overall. We speculate that, among patients infected with genotype 2b, only those with the IL28B minor variant might be treatment-refractory. That possibility might be validated further by a larger cohort study with genotype 2b.

The sustained virological response rates decreased significantly with failure of adherence to RBV (Table III), which was extracted as a factor associated with sustained virological response by univariate

analysis (Table IV). Regardless of the drug adherence, end of treatment response rates of patients infected with genotype 2 were around 94–99%, but the sustained virological response rates of the patients who received a total cumulative treatment dose of RBV of <80% was reduced significantly. As reported previously, increased RBV exposure during the treatment phase was associated with an increased likelihood of a sustained virological response [McHutchison et al., 2009] and these results confirm the importance of RBV in order to prevent relapse. Furthermore, host genetic variation leading to inosine triphosphatase (ITPA) deficiency protects against hemolytic anemia in chronic hepatitis C patients receiving RBV as revealed recently [Fellay et al., 2010]. We have reported also that the ITPA SNP, rs1127354, is confirmed to be a useful predictor of RBV-induced anemia in Japanese patients and that the incidence of early dose reduction was significantly higher in patients with ITPA-major (CC) variant as expected and, more importantly, that a significant higher sustained virological response rate was achieved in patients with the ITPA-hetero/minor (CA/AA) variant with non-genotype 1 or low viral loads [Sakamoto et al., 2010].

A rapid virological response was extracted in this study as a factor associated with sustained virological response only by multivariate analysis. It has been reported recently that a rapid virological response is an important treatment predictor and that drug adherence, which is reported to affect the therapeutic efficacy in patients infected with genotype 1, had no impact on the both sustained and rapid virological responses in combination therapy for patients infected with genotype 2 [Inoue et al., 2010]. The reasons why several host factors useful for predicting the response to therapy in patients with genotype 1, such as gender, age, progression of liver fibrosis and IL28B polymorphism had no influence on the efficacy in patients with genotype 2, can be attributed to IFN-sensitive genotypes. Similarly, the other viral factors useful for predicting the response to therapy, such as viral load and amino acid substitutions in the Core and NS5A regions had no influence on treatment outcomes. In this study, patients who achieved a rapid virological response had a high sustained virological response rate, regardless of IL28B polymorphism in patients with genotype 2a but, interestingly, none of the IL28B-TG and -GG patients with genotype 2b achieved a sustained virological response (although there were nine IL28B-TG and -GG patients with genotype 2b, two could not be determined as rapid virological response because the times at which they became HCV-negative were not recorded clearly, being described as 4–8 weeks.) These results also suggest that patients with both genotype 2b and IL28B minor allele are refractory cases.

IL28B encodes a protein also known as IFN- λ 3 [O'Brien, 2009]. IL28A (IFN- λ 2) and IL29 (IFN- λ 1) are found adjacent to IL28B on chromosome 19. These three IFN- λ cytokines, discovered in 2003 by two independent groups [Kotenko et al., 2003; Sheppard et al.,

2003] have been suggested to be involved in the suppression of replication of a number of viruses, including HCV [Robek et al., 2005; Marcello et al., 2006; Tanaka et al., 2010]. Humans have these three genes for IFN- λ , and this group of cytokines is now collectively referred to as type III IFN [Zhou et al., 2007]. IFN- λ functionally resembles type I IFN, inducing antiviral protection in vitro [Kotenko et al., 2003; Sheppard et al., 2003] as well as in vivo [Ank et al., 2006]. Type III IFN utilizes a receptor complex different from that of type I IFN, but both types of IFN induce STAT1, STAT2, and STAT3 activation by activation of a highly overlapping set of transcription factors, and the two types of IFN seem to have similar biological effects at a cellular level. Some in vitro studies have suggested that IFN- λ induces expression of IFN- λ genes [Siren et al., 2005]. Other in vitro studies also suggest that IFN- λ inhibits hepatitis C virus replication through a pattern of signal transduction and regulation of interferon-stimulated genes that is distinct from IFN- α and that the anti-HCV activity of either IFN- α or IFN- λ is enhanced by a low dose of the other [Marcello et al., 2006]. A novel mechanism of the interaction between IFN- α and IFN- λ may play a key role in the suppression of HCV [O'Brien, 2009].

In conclusion, IL28B polymorphism is predictive of PEG-IFN plus RBV treatment outcomes in patients infected with genotype 2, and more remarkably with genotype 2b. These results suggest that IL-28B polymorphism affects responses to IFN-based treatment in more difficult-to-treat subpopulations of HCV patients, and that intersubgenotypic differences between genotype 2a and 2b are revealed by responses to PEG-IFN plus RBV treatment according to IL28B variants.

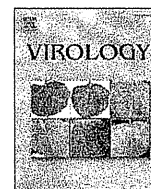
ACKNOWLEDGMENTS

The study is based on 10 multicenter hospitals throughout Japan, in the Kanto area (Tokyo Medical and Dental University Hospital, Musashino Red Cross Hospital, Kashiwa City Hospital, Kudanzaka Hospital, Showa General Hospital, Tsuchiura Kyodo General Hospital, Toride Kyodo General Hospital), Tokai area (Nagoya City University Hospital, Mishima Social Insurance Hospital) and Chugoku/Shikoku area (Ehime University Hospital).

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IL-6-mediated intersubgenotypic variation of interferon sensitivity in hepatitis C virus genotype 2a/2b chimeric clones

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ABSTRACT

Mechanisms of difference in interferon sensitivity between hepatitis C virus (HCV) strains have yet to be clarified. Here, we constructed an infectious genotype2b clone and analyzed differences in interferon-alpha sensitivity between HCV-2b and 2a-JFH1 clones using intergenotypic homologous recombination. The HCV-2b/JFH1 chimeric virus able to infect Huh7.5.1 cells and was significantly more sensitive to IFN than JFH1. IFN-induced expression of MxA and 25-OAS was significantly lower in JFH1 than in 2b/JFH1-infected cells. In JFH1-infected cells, expression of SOCS3 and its inducer, IL-6, was significantly higher than in 2b/JFH1-infected cells. The IFN-resistance of JFH1 cells was negated by siRNA-knock down of SOCS3 expression and by pretreatment with anti-IL6 antibody. In conclusion, intergenotypic differences of IFN sensitivity of HCV may be attributable to the sequences of HCV structural proteins and can be determined by SOCS3 and IL-6 expression levels.

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Introduction

Hepatitis C virus (HCV) is one of the most important pathogens causing liver-related morbidity and mortality (Alter, 1997). There is no therapeutic or prophylactic vaccine available for HCV and type I interferons have been the mainstay of HCV therapeutics (Hoofnagle and di Bisceglie, 1997). Antiviral therapeutic options against HCV are limited and yield unsatisfactory responses (Fried et al., 2002). Given these situations, gaining a detailed understanding of the molecular mechanisms of interferon resistance has been a high priority in academia and industry.

Molecular studies of HCV have been hampered by the lack of efficient *in vitro* and *in vivo* models of infection, which has been partly overcome by the development of HCV subgenomic replicons (Blight et al., 2000; Kato et al., 2003; Lohmann et al., 1999) and the HCV-JFH1 cell culture system

(Wakita et al., 2005). HCV-JFH1 is an isolate of HCV genotype 2a that was obtained from a patient with fulminant hepatitis C. The full-length JFH1 genome has been shown to produce infectious particles in cell culture. Simultaneously, a robustly replicating intragenotypic chimera has been reported, which consists of the structural region of a genotype 2a, J6-clone and nonstructural region of JFH-1 (Lindenbach et al., 2005).

HCV isolates are classified into seven major genotypes and multiple subtypes (Gottwein et al., 2009). In infected individuals, HCV exists as quasispecies of closely related genomes (Bukh et al., 1995). A number of studies have suggested that the outcome of HCV infection, as well as the response to interferon treatment, depends on the genotype or quasispecies with which the patient is infected. However, it is not clear how these subtle genetic differences of HCV affect viral replication, infectivity and host responses. Thus, it is important to establish multiple cell culture-permissive strains of different genotypes and isolates of the same genotype for their potential value for characterizing the virus life cycle, drug sensitivity and virus-related cell signaling.

Our present work describes the generation of chimeric viruses with their structural regions from genotype 2b and non-structural genes from the HCV-JFH1 strain. The intergenotypic 2b/JFH1 viruses were compared in terms of intracellular replication, infectious virus production and sensitivity to interferon-alpha. Here we show that the differences in sensitivity to interferon are attributable to upregulated expression of the cellular interferon signal attenuator, SOCS3, and that this upregulation is caused by overexpression of interleukin-6 (IL-6).

Abbreviations: HCV, hepatitis C virus; TLR, toll-like receptor; FBS, fetal bovine serum; ISG, interferon-stimulated gene; IFN, interferon; SOCS, suppressor of cytokine signaling; IL, interleukin; ALT, alanine aminotransferase; UTR, untranslated region; CLEIA, chemiluminescence enzyme immunoassay; PVDF, polyvinylidene fluoride; STAT, signal transducer and activator of transcription; IFNAR, interferon alpha/beta receptor.

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Results

In vitro and *in vivo* infectivity analyses of HCV-2b and 2b/JFH1 intragenotypic chimeras

First, we investigated the infectivity of the full-length genotype 2b clone *in vitro* and *in vivo*. The full-length genotype 2b HCV clone was infectious after direct injection of RNA transcribed *in vitro* into the livers of human hepatocyte engrafted albumin-uPA/SCID mice (see the Supplementary Fig. 1). However, transfection of the HCV RNA into Huh7.5.1 cells did not lead to replication or secretion of virions. Knowing that the full-length genotype 2b HCV was not infectious *in vitro*, we constructed genotype2b/JFH1 intergenotypic recombinants. We constructed three recombinant clones of 2b/JFH1 (Fig. 1A), which were joined between E2 and p7 (JE31F), NS2 and NS3 (JE39F), and within NS2 at nt. 2867 (JEC3F). After transfection of these chimeric HCV RNAs and JFH-1 RNAs into Huh7.5.1 cells, all four clones expressed detectable amounts of HCV core protein in the cells (Fig. 1B) and culture fluid (Fig. 1C). Among the four clones, JEC3F produced the highest level of core protein in the cells and culture fluid. Similarly, in the reinfection assays, JEC3F infected naïve cells most efficiently (Figs. 1D and E). We then compared the infectivity of JEC3F with the other chimeric viruses, genotype2a J6/JFH1 and the JFH1 clone (Supplementary Fig. 2). Transfection of the individual clones into Huh7.5.1 cells showed that JEC3F and the 2b/JFH1 chimera secreted core protein into the medium most efficiently (Fig. 1C). We measured HCV core antigen and HCV-RNA levels in culture supernatant of JEC3F and JFH-1 infected cells. As shown in Fig. 1F, the ratio between supernatant HCV core antigen and HCV-RNA between JEC3F and JFH1 was well correlated each other.

Comparisons of sensitivity to IFN between intragenotypic chimeras and JFH1

Next, we investigated the interferon-alpha sensitivity of the three 2b/JFH1 chimeric viruses with different junctions, JE31F, JE39F and JEC3F, as well as JFH1. The four viral RNAs were transfected separately into Huh7.5.1 cells and were treated with 0, 1, 3 or 9 IU/mL of interferon-alpha-2b. Seventy-two hours after addition of interferon, core antigen was measured in the culture fluid. As shown in Fig. 2, all 2b/JFH1 chimeric clones showed significantly higher responses to interferon than JFH1 ($p < 0.01$). These results indicate that the relative interferon sensitivity of 2b/JFH1 clones over JFH1 could be attributable to the sequences of HCV-2b-derived structural proteins, especially core, E1 or E2 protein.

Expression of IFN stimulated genes and STAT1 and 2 phosphorylation in HCV-infected cells

Knowing that the 2b/JFH1 chimeric clones are more sensitive to interferon than JFH1, we next analyzed the effects on cellular interferon signaling. We investigated the expression levels of the interferon-stimulated genes (ISGs), 250AS and MxA mRNAs that mediate antiviral effects (Itsui et al., 2009; Itsui et al., 2006). Induction of 250AS and MxA by IFN was significantly suppressed in cells infected with HCV-JFH1 and the JEC3F clones. Of note was that the induction of these ISGs was suppressed substantially in JFH1-infected cells compared to JEC3F-infected cells (Figs. 3A and B). We then detected IFN-induced phosphorylation of STAT1 and STAT2 to pSTAT1 and pSTAT2 in uninfected and JFH1- and JEC3F-infected cells. Phosphorylation of STAT1 and STAT2 occurs within minutes after addition of IFN and substantially decreased at time points later than 8 hours [Itsui, 2006 #1025]. Thus, we detected pSTAT1 and pSTAT2 before and at 15 minutes after IFN treatment. As shown in Figs. 3C and D, production of pSTAT1 and pSTAT2 was decreased substantially in JFH1-infected cells, compared with uninfected and JEC3F-infected

cells. These finding indicated that the differences in sensitivity to interferon of JFH1 and JEC3F were closely associated with attenuation of the cellular IFN signaling pathway.

SOCS 3 is up-regulated in JFH-infected, IFN-resistant cells

We next investigated the effects of HCV replication on the expression of SOCS1 and SOCS3 that suppress IFN receptor-mediated signaling (Song and Shuai, 1998; Vlotides et al., 2004). While SOCS1 mRNA expression did not differ significantly between uninfected and JFH1- and JEC3F-infected cells, the SOCS3 mRNA expression level was significantly higher in JFH1-infected cells than in uninfected and JEC3F-infected cells (Figs. 4A and B).

Knock down of the SOCS3 gene

To verify that SOCS3 was the key molecule determining the sensitivity to IFN, we performed siRNA knock down of SOCS3 in the virus-infected cells. A SOCS3-directed siRNA was cotransfected with HCV-JFH1 or -JEC3F RNA into Huh7.5.1 cells. Three days after transfection we measured SOCS3 mRNA expression in JFH1 and JEC3F-transfected cells with or without SOCS3-siRNA. Interestingly, SOCS3-knock down in JFH1-transfected cells restored sensitivity of IFN to the same levels as JEC3F-transfected cells (Figs. 5A and B).

Interleukin-6 is involved in SOCS-mediated interferon resistance

It has been reported that SOCS3 is induced principally by phosphorylated STAT3 (pSTAT3) (Hanada et al., 2003) and that interleukin-6 (IL-6) is a strong inducer of pSTAT3 via receptor-mediated Janus kinase activation in the liver (Ramadori and Christ, 1999). This background led us to investigate whether overexpression of SOCS3 is associated with overproduction of IL-6. We investigated Phosphorylated STAT3 (pSTAT3) expression and IL-6 mRNA expression in JFH1- and JEC3F-transfected Huh7.5.1 cells. Phosphorylated STAT3 level was significantly higher in JFH1-transfected cells than JEC3F-transfected cells and naïve Huh7.5.1 cell (Fig. 6A). Moreover IL-6 gene expression level was significantly higher in JFH1-transfected cells than JEC3F-transfected cells (Fig. 6B). Consistent with previous reports, treatment of the Huh7.5.1 cells with IL-6 induced expression of SOCS3 and SOCS1 mRNAs with SOCS3 being much stronger than SOCS1 (Fig. 6C).

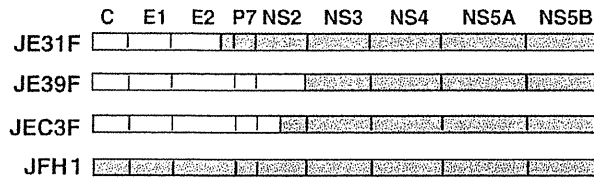
Anti-IL-6 antibody restored IFN-resistance to HCV-infected cells

To investigate whether IL-6 is responsible for HCV infection-induced upregulation of SOCS and for resistance to interferon, JFH1 and JEC3F-infected Huh7.5.1 cells were pretreated with antibodies directed against IL-6 and subsequently treated with interferon. Interestingly, anti-IL-6-treated HCV-infected cells became significantly more susceptible to IFN treatment (Fig. 6D) without affecting viral expression levels in the absence of interferon (Fig. 6E). Cellular levels of SOCS3 mRNA were significantly lower in anti-IL-6-treated cells than untreated cells (Fig. 6F). These results strongly suggested that the interferon resistance of HCV-infected cells and the difference between the two viral strains are partly mediated by internal overproduction of IL-6 and subsequent upregulation of SOCS3.

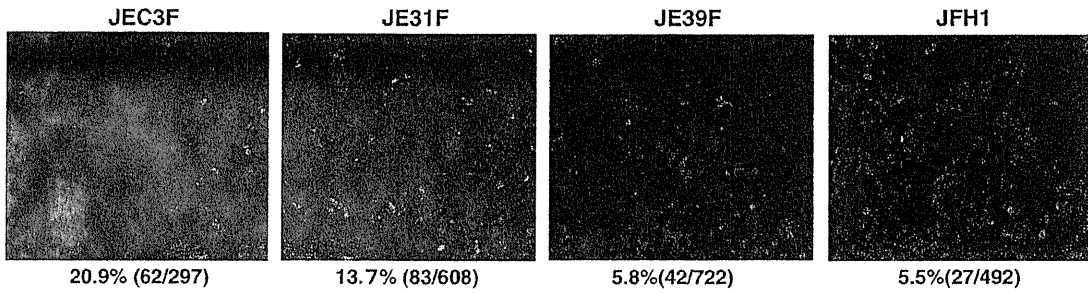
Determination of the HCV structural region that induced SOCS3 and IL6

We studied further which part of HCV structural polyprotein is responsible for the difference in interferon-sensitivity. We constructed two additional chimeric clones between HCV-2b and JFH1. The 2bCoreJFH1 had the 2b-core region followed by the JFH1-structural and nonstructural regions. JCoreC3F was derived from JEC3F by exchanging the 2b-core with the JFH1-core (Fig. 7A). As

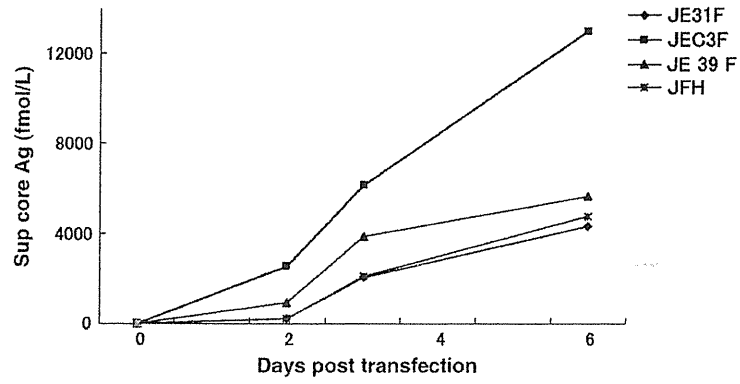
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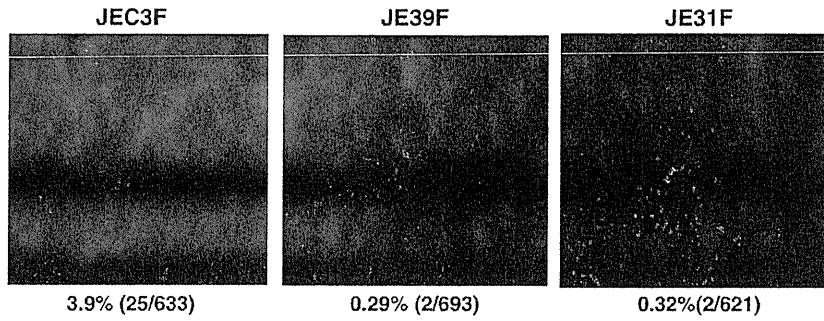
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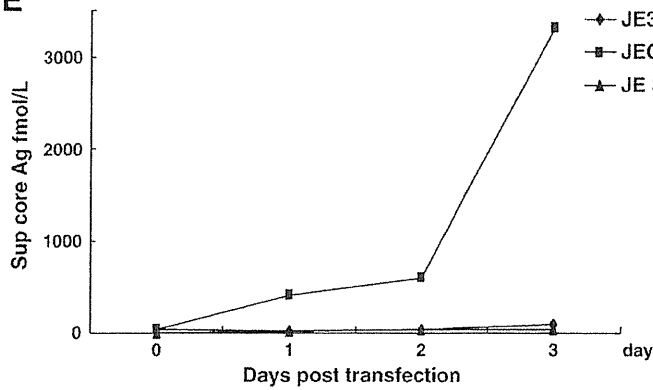
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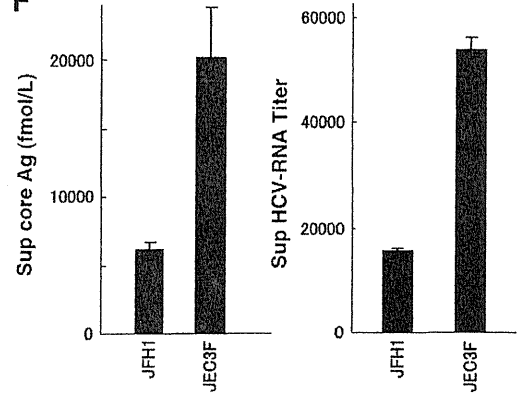
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shown in Fig. 7B JFH1 and JCoreC3F, which had a JFH1-derived core region, were significantly more resistant to IFN than JEC3F and 2bCoreJFH1, with a 2b-derived core (Fig. 7B). Consistent with the interferon sensitivity results, JFH1 and JcoreC3F-infected cells expressed SOCS3 and IL6 mRNAs at significantly higher levels than JEC3F and 2bCoreJFH1-infected cells (Figs. 7C and D). These differences in gene expression were inversely associated with the cellular expression levels of each HCV chimeric clone (Fig. 7E). These results indicate that the amino acid sequence of the core protein is responsible for IL-6 and SOCS3-mediated interferon resistance.

Discussion

In this study, we succeeded in establishing a new genotype 2b infectious HCV clone and genotype 2b/JFH1 cell culture-competent intragenotypic chimeric viruses (Fig. 1). Relative interferon sensitivities of 2b/JFH1 chimeras, compared with HCV-JFH1 virus (Fig. 2), led us to conduct a series of assays to investigate the molecular mechanisms of IFN-related response pathways. We found that IFN-alpha receptor-mediated cellular responses were more attenuated in HCV-JFH1- and 2b/JFH1 chimera-infected than in uninfected Huh7.5.1 cells, but more potently for HCV-JFH1. Precise intragenotypic recombination analyses showed that the amino acid sequence of the HCV core protein is responsible for the differences in interferon sensitivity (Figs. 2, 7). The differences in the interferon-mediated antiviral effects were demonstrated further by the different rates of induction of interferon-inducible MxA and 25-OAS mRNAs (Figs. 3A and B) and IFN induced phosphorylation of STAT1 and STAT2 (Figs. 3D and E). We have demonstrated further that the expression of an interferon signal attenuator, SOCS3, was significantly higher in JFH1 than in 2b/JFH1-infected cells (Song and Shuai, 1998; Vlotides et al., 2004). Indeed, the siRNA-knock down of SOCS3 in JFH1 and 2b/JFH1-infected cells resulted in responsiveness to IFN (Fig. 5). Moreover, cellular expression of IL-6, which increases cytoplasmic phospho-STAT3 (Fig. 6A) and induces SOCS3 expression (Ramadori and Christ, 1999) was significantly higher in JFH1 transfected cells (Fig. 6B). Furthermore, by pre-treatment with anti-IL-6 antibody, JFH1- and 2b/JFH1-infected cells partially recovered elevation of SOCS3 expression and unresponsiveness to IFN (Fig. 6D). Taking all these things together, it is strongly suggested that the differences in IFN sensitivity between genotypes or isolates could be explained by SOCS3-mediated attenuation of interferon responses and, more importantly, IL-6 may constitute a molecular target to reverse such cellular interferon resistance.

Vast numbers of studies have failed to construct infectious HCV clones, other than HCV-JFH1. Murayama, et al. have conducted intragenotypic homologous recombination analyses between HCV-J6 and -JFH1 and have reported that the NS3 protease and NS5B polymerase are essential for replication of the recombinant virus (Murayama et al., 2007). Up to now, several JFH1-based chimeric viruses have been reported, which include genotypes 4a (Scheel et al.,

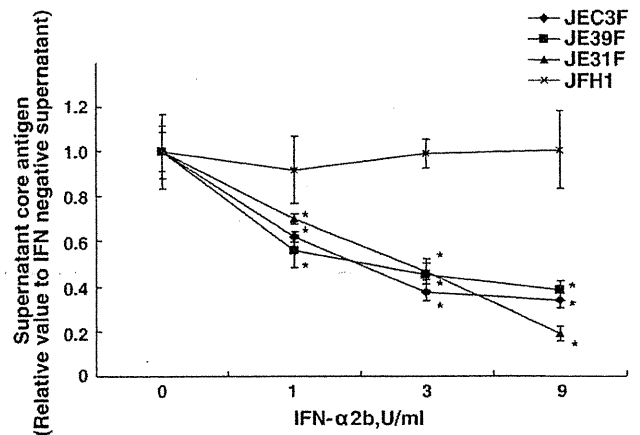


Fig. 2. Comparison IFN-alpha sensitivity among 2b/JFH1 chimeric viruses and JFH1. Ten μ g of JE31F, JE39F, JEC3F, JFH1 RNA were transfected into 5×10^6 Huh7.5.1 cells. The transfected cells were divided into 12 wells. Forty eight hours after transfection, cells were washed twice with PBS and treated with 0, 1, 3 and, 9 U/ml IFN-alpha-2b. Seventy-two hours after IFN-alpha 2b addition, quantification of HCV core antigen in culture fluids was conducted. The experiments were conducted twice by using Huh 751 cells of different passage, and a representative data was shown. Assays were done in triplicate and the data are shown as mean \pm sd. Asterisks indicate p-values of less than 0.05.

2008), genotype 1a, 1b, 2a (Pietschmann et al., 2006), genotype 3a (Gottwein et al., 2007), genotype 5 a (Jensen et al., 2008) and, genotype 2b, 6a, 7a (Gottwein et al., 2009). Gottwein, et al. constructed intergenotypic chimeric HCV from JFH1 and genotypes 1 through 7 and analyzed differences in sensitivity to antiviral drugs (Gottwein et al., 2009). However, intergenotypic differences in sensitivity to IFN-alpha and the molecular mechanisms involved have not been well characterized. In this study, we constructed several chimeric virus clones between HCV-2b and HCV-JFH1 (2a), which showed variable sensitivity to IFN and confirmed that the core region is responsible for such IFN sensitivity. This study may support the feasibility of such inter and intragenotypic homologous recombination approaches to characterize differences in viral kinetics and drug responses.

Type I IFNs and their responsive ISGs are the principal mediators of host defense against virus infections, including HCV (Chang et al., 1991; Kalvakolanu, 2003; Ronni et al., 1998). On binding of IFNs to their receptors, IFNAR1 and IFNAR2, Janus kinases-1 and -2 phosphorylate STAT1 and STAT2 to form ISGF-3, which translocates to the nucleus and activates transcription of ISGs (Samuel, 2001; Taniguchi et al., 2001; Taniguchi and Takaoka, 2002). Members of the SOCS family are potent inhibitors of type I and type III IFN-induced activation of the Jak-STAT pathway and subsequent expression of ISGs (Vlotides et al., 2004). In HCV subgenomic replicon-expressing cells, expression levels of SOCS3 were inversely correlated with sensitivity to IFN to suppress viral RNA replication (Zhu et al., 2005).

Fig. 1. Replication and infection competency of HCV-2b/JFH1 chimeric viruses. A. Genomic structures of HCV-JFH1, HCV-2b and 2b/JFH1 chimeric viruses. Intergenotypic homologous recombination was conducted between the HCV-2b and JFH1 (2a) clones and three chimeric clones were constructed that were joined between NS2-NS3 (JE39F), and within E2 at nt2541 (JE31F) and NS2 at nt. 2867 (JEC3F). B. Immunocytochemistry of HCV core. HCV RNA-transfected Huh7.5.1 cells were plated onto 22 mm-round micro cover glasses. Immunocytochemistry was performed 4 days after transfection using mouse-anti-core antibody (green) and DAPI (blue). C. Time courses of 2b/JFH1- and JFH1-transfected cells. *In vitro* transcribed HCV RNAs were transfected into Huh7.5.1 cells by electroporation and HCV core levels of culture fluids were sampled at the time points indicated and core antigen levels were measured. The experiment was done three times with similar results independently. Panel C shows representative data. D. Immunocytochemistry of HCV core. HCV RNA-infected Huh7.5.1 cells using Panel B supernatant that have same amount of HCV core antigen were plated onto 22 mm-round micro cover glasses. Immunocytochemistry was performed 4 days after infection using mouse-anti-core antibody (green) and DAPI (blue). Numbers at the bottom denote percentages of HCV core-positive cells. E. Time courses of 2b/JFH1 infected cells. JE31F, JE39F, JEC3F RNA-transfected cell culture fluids were used to infect naive Huh7.5.1 cells in 60 mm-diameter plates at density of 3×10^5 cells per plate. Quantification of HCV core antigen in culture supernatants was carried out at 24 hours, 48 hours, 72 hours and 144 hours after inoculation. The experiment was done three times with similar results independently. Panel E shows representative data. F. Comparison between JFH1 and JEC3F supernatant HCV-RNA titer and core antigen. Four days after JFH1 and JEC3F RNA transfection, culture supernatant was harvested and subjected to both HCV core antigen assay and realtime RT-PCR of HCV-RNA. Assays were done in triplicate and the data are shown as mean \pm sd.

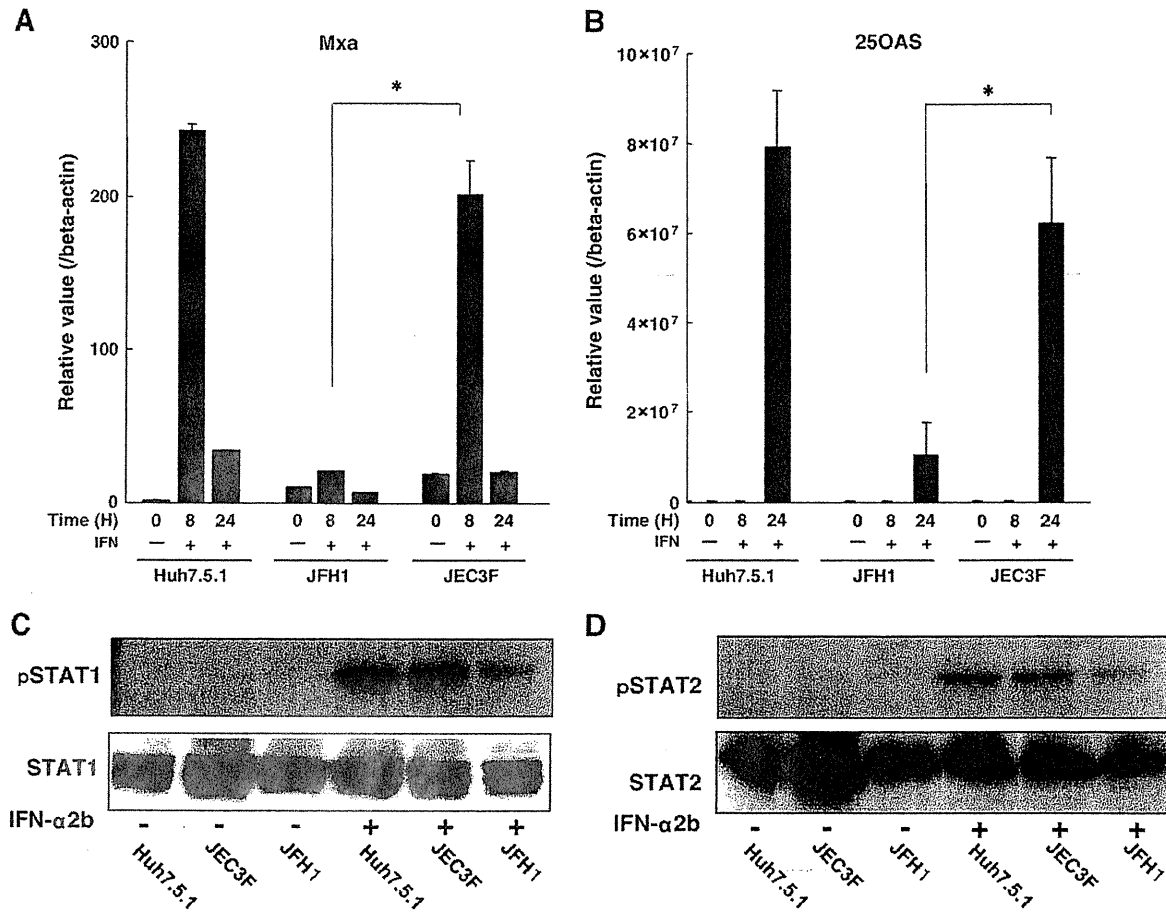


Fig. 3. Induction by interferon of the interferon-inducible genes, MxA (panel A), 25-OAS (panel B) and phosphorylated STAT1 (panel C) and STAT2 (panel D). JEC3F and JFH1 10 μ g RNA was transfected into Huh7.5.1 cells. Forty-eight hours after transfection, the cells were treated with 25 U/ml IFN- α 2b. Total cellular RNA was isolated before and 8 and 24 hours after IFN treatment. Relative gene expression levels of MxA (panel A) and 25-OAS (panel B) were determined by real-time PCR at the time points indicated. JEC3F and JFH1 RNA and MOCK was transfected into Huh7.5.1 cells. Forty eight hours after transfection, the cells were treated with 25 U/ml IFN- α 2b. Total cellular protein was isolated before and 15 minutes after IFN treatment. Ten μ g of extracted protein were used for analysis of phosphorylated STAT1, STAT2 protein and STAT1, STAT2 protein as controls. Assays were done in triplicate and the data are shown as mean \pm sd. Asterisks indicate p-values of less than 0.05.

HCV, on the other hand, counteracts such IFN-mediated antiviral pathways. The NS5A and E2 proteins interfere with the action of IFN by inhibiting the activity of PKR (He and Katze, 2002; Taylor et al., 1999). NS5A also induced expression of IL-8 and attenuated expression of ISGs (Polyak et al., 2001). HCV core protein has been reported to bind the STAT1-SH domain (Lin et al., 2006) or destabilize STAT1 (Lin et al., 2005) to block IFN signaling. It also has been reported that overexpression of core protein upregulated SOCS3 expression (Bode et al., 2003). In this study, we used full-length HCV cell culture and found, for the first time, that SOCS3 expression is upregulated differently depending on the genetic sequences of HCV strains and that these differences in SOCS3 expression are associated with sensitivity to IFN. Moreover, overexpression and knock down of SOCS3 expression were closely associated with the IFN sensitivity of the HCV-infected cells. These results indicate that interferon-resistance of HCV-infected cells is directed by overexpression of SOCS3, which may be upregulated by HCV proteins as reported (Bode et al., 2003) (Kawaguchi et al., 2004). A sequence comparison of our HCV2b and JFH1 clones has found 16 amino acid differences. These structural differences of the core protein might affect cellular responses to interferon (see the Supplementary Fig. 4).

It has been reported that IL-6 is the principal activator of STAT3 in hepatocytes through binding its receptor (Hanada et al., 2003; Ramadori and Christ, 1999). Furthermore, plasma IL-6 levels are elevated in

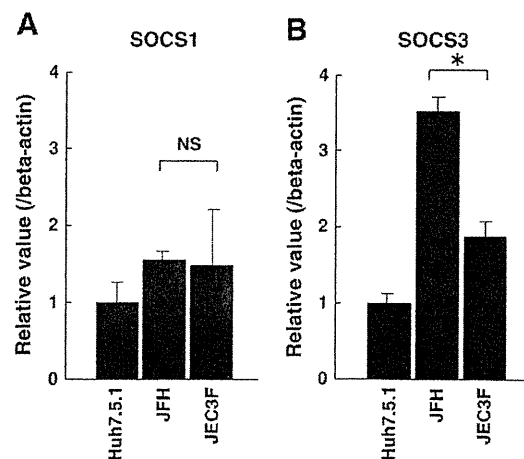


Fig. 4. Expression of SOCS1 mRNA (panel A), SOCS3 mRNA (panel B). Forty-eight hours after transfection of JEC3F, JFH1 10 μ g RNA or mock transfection into Huh7.5.1 cells, total RNA and total protein were isolated. Relative gene expression levels of SOCS1 (panel A) and SOCS3 (panel B) and were determined by real time PCR. Values are shown as relative to those of uninfected Huh 751 cells. Assays were done in triplicate and the data are shown as mean \pm sd. Asterisks indicate p-values of less than 0.05.

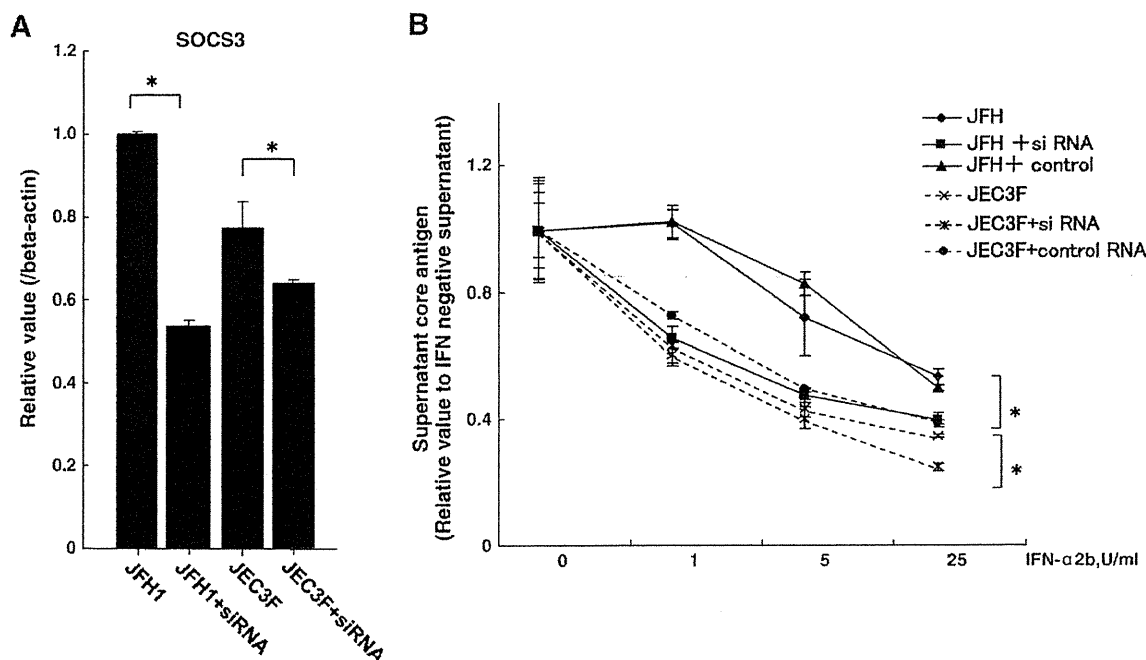


Fig. 5. Differences in sensitivities to IFN between SOCS3-knock down, HCV transfected cells. JFH1 or JEC3F 10 μ g RNA, and 80 pmol siRNA SOCS3-HSS113312 or MOCK were electroporated into 5×10^6 uninfected Huh7.5.1 cells. A. Expression of SOCS3 mRNA in uninfected and HCV-infected Huh7 cells. Forty-eight hours after transfection, total RNA was isolated. Relative gene expression level of SOCS3 were determined by real time PCR. Values are shown as relative to those of JFH1 infected Huh 751 cells. Assays were done in triplicate and the data are shown as mean \pm sd. Asterisks indicate p-values of less than 0.05. B. Dose-dependent suppression of HCV replication by IFN in SOCS3-knock-down, HCV-infected cells. The above siRNA and HCV RNA-transfected cells were divided into 12 wells. Forty eight hours after transfection, the cells were treated with 0, 1, 5 and 25 U/ml of IFN- α 2b. Seventy two hours after treatment, quantification of HCV core antigen in culture fluids was carried out. Assays were done in triplicate and the data are shown as mean \pm sd. Asterisks indicate p-values of less than 0.05.

chronic hepatitis C patients (Malaguarnera et al., 1997). Consistent with those reports, we found that IL-6 strongly induced SOCS3 expression in Huh7.5.1 cells (Fig. 6C). More importantly, cellular IL6 expression levels were in the order of uninfected < JEC3F << JFH1-infected cells, which correlated well with SOCS3 expression (Fig. 4) and with cellular responses to IFN (Fig. 2). In addition, the IFN-resistant JcoreC3F, in which the core region of JEC3F had been re-substituted by the JFH1-core, induced comparatively higher levels of IL-6 and SOCS3 mRNA to JFH1 (Fig. 7). Taken together, our results indicate that the amino acid sequence of the core protein determines IL-6 and SOCS3 expression levels and, as a consequence, resistance to IFNs.

It remains to be clarified what are the inducers of IL-6. There are reports that HCV core protein activates toll-like receptor (TLR)-2 in Huh7 cells and in adult human hepatocytes (Hoffmann et al., 2009; Mozer-Lisewska et al., 2005). TLRs are known to activate downward NF-kappaB signaling that upregulates IL-6 expression. Alternatively, IL-6 may be secreted in response to cellular steatosis and insulin resistance. HCV patients with obesity or insulin resistance are refractory to IFN treatments. Such patients have higher levels of hepatic SOCS3 expression than those without obesity or insulin resistance (Miyaaki et al., 2009; Walsh et al., 2006). More recently, Sabio, *et al* have reported that fatty acid-induced secretion of IL-6 from adipocytes upregulates hepatic SOCS3, leading to insulin-resistance (Sabio et al., 2008).

In conclusion, our study demonstrates that HCV intragenotypic and inter-strain differences in IFN sensitivity can be, in most part, attributable to the amino acid sequence of the HCV core protein and that such IFN sensitivities are determined by cellular expression levels of SOCS3 and IL-6. Therapeutic targeting of IL-6 potentially may be a key to targeting IFN-resistance and improving antiviral chemotherapeutics against HCV.

Materials and Methods

Reagents and antibodies

Recombinant human interferon alpha-2b was from Schering-Plough (Kenilworth, NJ). Anti-CD 81 antibody (JS-81) was from BD Biosciences (Franklin Lakes, NJ) (Morikawa et al., 2007), anti-IL6 receptor antibody was from Chugai pharmaceutical Co (Tokyo, Japan), anti-SOCS3 was from Cell Signaling (Beverly, MA), and anti-IL6 antibody was from R&D Systems (Minneapolis, MN).

Cloning of HCV cDNA from patient serum

A serum sample was obtained from a 32-year-old male who developed acute hepatitis after intravenous drug injection. Serum was obtained one week after the onset of symptoms. Total RNA was extracted from 150 μ l of serum using ISOGEN (Nippon Gene, Osaka, Japan). cDNA was synthesized using SuperScript II (Invitrogen, Carlsbad, CA) reverse transcriptase. PCR primers, based on a genotype 2b prototype sequence, HC-J8 (accession number: D10988), were used to amplify 14 fragments of HCV cDNA covering nt. 13–9478 (nucleotide numbers corresponded to HC-J8) by PCR. All amplicons were purified and cloned into the pGEM-T EASY vector (Promega, Madison, WI) and nucleotide sequences were determined using Big Dye Terminator Cycle Sequencing Ready Reaction kits (Applied Biosystems, Foster City, CA) and an automated DNA sequencer (ABI PRISM $\text{\textcircled{R}}$ 310 Genetic Analyzer; Applied Biosystems). The consensus sequence of five clones was adopted for each region. Each consensus sequence segment of HCV was assembled into pJFH1-full (Wakita et al., 2005) by substituting the insert sequence of pJFH1-full.

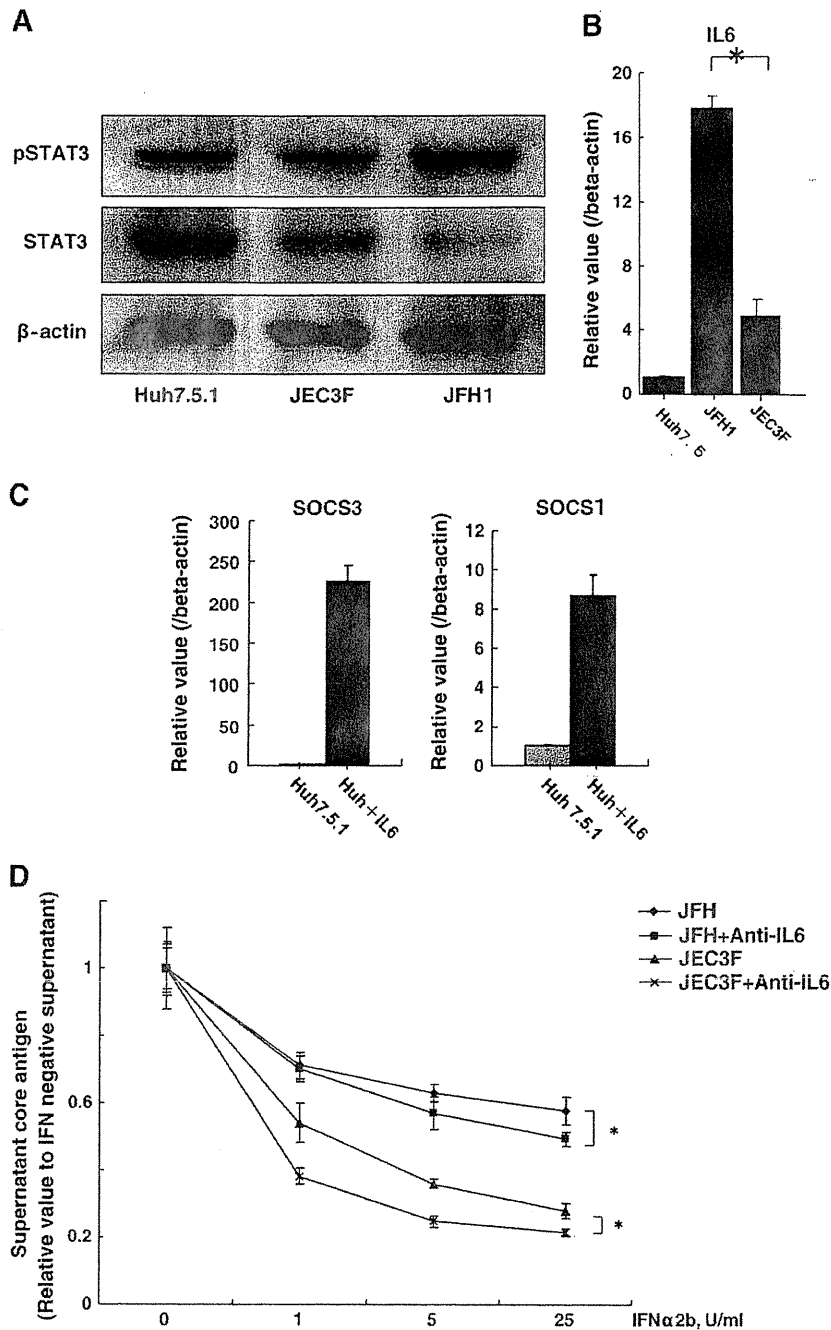


Fig. 6. IL-6 expression in HCV infected cells and change in IFN sensitivity by treatment with anti-IL6 antibody. **A.** Expression of cytoplasmic phospho-STAT3 in uninfected and HCV-infected Huh7 cells. JEC3F, JFH1 10 μ g RNA and MOCK was transfected into Huh7.5.1 cells. Forty eight hours total cellular protein was isolated. Ten μ g of extracted protein were used for analysis of phosphorylated STAT3, STAT protein and β -actin as controls. **B.** Expression of Interleukin-6 mRNA in uninfected and HCV-infected Huh7 cells. Forty-eight hours after transfection, total RNA was isolated. Relative gene expression level of IL6 were determined by real time PCR. Values are shown as relative to those of uninfected Huh 751 cells. Assays were done in triplicate and the data are shown as mean \pm sd. **C.** IL-6 induces SOCS3 strongly in uninfected Huh7.5.1 cells. Uninfected Huh7.5.1 cells were treated with 10 ng/ml recombinant human IL6 (PEPRO TEC EC, London, England). Fifteen minutes after treatment, total RNA was isolated. Relative gene expression levels of SOCS1 and SOCS3 were determined by real time PCR. Uninfected Huh7.5.1 cells that were not treated with IL6 were used as a control. Values are shown as relative to those of uninfected Huh 751 cells. Assays were done in triplicate and the data are shown as mean \pm sd. **D.** Dose-dependent suppression of HCV replication by IFN in HCV-infected cells pre-treated with anti-IL-6 antibody. Immediately after electroporation, HCV RNA-transfected cells were divided into 12 wells and pretreated with 1 μ g/ml anti-IL-6 antibody. Forty eight hours after transfection, the cells were washed with PBS and treated with 0, 1, 5 and 25 U/ml of IFN-alpha 2b. Seventy two hours after treatment, quantification of HCV core antigen was carried out in culture fluids. Assays were done in triplicate and the data are shown as mean \pm sd. **E.** Core protein secretion levels following treatment of HCV-transfected cells with anti-IL-6 antibody. After treatment with anti-IL-6 antibody, HCV RNA-transfected cells were divided into 12 wells. Five days after transfection, quantification of HCV core antigen was carried out in culture fluids. Assays were done in triplicate and the data are shown as mean \pm sd. **F.** Expression of SOCS3 mRNA in uninfected and HCV-infected Huh7 cells. Forty-eight hours after transfection, total RNA was isolated. Relative SOCS3 gene to beta-actin gene expression were determined by real time PCR. Values are shown as relative to those of uninfected Huh 751 cells. Assays were done in triplicate and the data are shown as mean \pm sd. Asterisks indicate p-values of less than 0.05.