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ORIGINAL ARTICLE

Prolongation of interferon therapy for recurrent hepatitis C after living donor liver transplantation: Analysis of predictive factors of sustained virological response, including amino acid sequence of the core and NS5A regions of hepatitis C virus

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Abstract

Objective. The aim of the present retrospective study was to evaluate the therapeutic efficacy and predictive factors of prolongation of treatment with peginterferon (PEGIFN) combined with ribavirin (RBV) for recurrent hepatitis C after living donor liver transplantation (LDLT). ***Methods.*** Fifty-three patients underwent LDLT due to HCV-related end-stage liver disease. Sixteen patients were removed from the study as a result of early death ($n = 14$), no recurrence of HCV ($n = 1$) and refusal of antiviral therapy ($n = 1$). Therapy is ongoing in another 10 patients. The remaining 27 patients were available to establish the efficacy of IFN therapy. HCV genotype was 1b in 24 patients. All patients with genotype 1b were treated with IFN therapy for at least 48 weeks after HCV RNA levels had become undetectable. Amino acid substitutions in the HCV core region and NS5A region were analyzed by direct sequencing before LDLT. ***Results.*** The rate of sustained virological response (SVR) was 37.0% (10/27). SVR rate in patients with genotype 1 was 29.2% (7/24) and 100% (3/3) in patients with genotype 2. Most patients with genotype 1b whose HCV RNA reached undetectable levels achieved SVR (87.5%; 7/8). However, mutation of the HCV core region and number of ISDR mutations were not associated with SVR rate in LDLT in our study. ***Conclusions.*** Prolonged IFN therapy for more than 48 weeks after HCV RNA reached undetectable levels might prevent virological relapse of HCV.

Key Words: *Core and NS5A regions, HCV, IFN, LDLT*

Introduction

Hepatitis C virus (HCV)-related end-stage liver disease is currently the leading indication for liver transplantation (LT). Unfortunately, prevention of HCV infection after transplantation is difficult and, unlike the situation with the prevention of hepatitis B virus after transplantation [1], HCV re-infection after LT is almost universal, with histological evidence of chronic hepatitis in approximately 50% of patients within 1 year and cirrhosis in about 30% after 5 years. This in turn yields an excess risk of death or

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retransplantation for liver failure 10–15 years after transplantation [2]. Given this risk, it seems reasonable to offer antiviral therapy to liver transplant recipients. Many reports have noted rates of sustained viral response (SVR) ranging from only 10% to 30% in liver transplant recipients with recurrent HCV treated for 48 weeks [3–10], indicating the need for new treatment regimens with higher SVR rates. Recent reports have indicated that the extension of treatment with peginterferon and ribavirin (PEGIFN/RBV) from 48 to 72 weeks significantly increases the rate of SVR in immunocompetent patients, particularly slow virological responders [11,12], and one report noted that extended treatment for recurrent hepatitis C infection after liver transplantation (LT) was effective [13,14].

There are many predictive factors of successful treatment with the combination of peginterferon and ribavirin in immunocompetent patients, including the viral factors, such as HCV genotype, pretreatment viral load, amino acid (aa) 70 and/or 91 in the HCV core protein, amino acid substitutions in the HCV NS5A region [15–17]. In their multivariate analyses of predictors of SVR, Akuta and colleagues identified substitutions of aa 70 and 91 in the HCV core region (double-wild-type; odds ratio 5.988) as predictive [18], whereas Enomoto identified substitutions of amino acids of the HCV NS5A region (mutant type; odds ratio 5.3) as predictive [15].

With regard to length of treatment, one study reported that an early viral response at 3 months was useful in predicting a lack of response to antiviral therapy in liver transplant recipients with recurrent hepatitis C [19,20]. To our knowledge, however, no study has analyzed viral factors in extended treatment for recurrent hepatitis C infection after liver transplantation.

The aim of the present study was to evaluate the therapeutic efficacy of peginterferon in combination with ribavirin (PEGIFN/RBV) on long-term treatment for recurrent hepatitis C after LDLT, and predictive factors of virological response to this treatment, particularly viral factors. This study is first report of predictive factors associated with virological response in recurrent hepatitis C patients after LDLT, including amino acid substitutions in the core region and NS5A region.

Material and methods

Patients

A total of 53 patients who underwent LDLT due to HCV-related end-stage liver disease from 2000 to January 2009 were enrolled for this retrospective study. Among them, 14 patients died before the start of therapy, 1 refused treatment with antiviral therapy, and 1 did not become positive for HCV RNA after LDLT. Eventually, leaving 37 patients treated with PEGIFN/RBV in our institution. Of these, 10 patients are currently continuing antiviral therapy.

We introduced all patients to IFN therapy in principle. The efficacy of IFN therapy could thus be established in 27 patients (Figure 1).

Antiviral treatment protocol

Patients received 1.5 μg/kg body weight (BW) PEGIFN (Peg-Intron; Schering-Plough, Segrate, Italy) subcutaneously (s.c.) once weekly and 200 mg RBV (Rebetol; Schering-Plough). PEGIFN/RBV was continued for more than 1 year after serum HCV RNA becomes negative. At the end of active treatment, the patients were followed for further 24 weeks without treatment.

The study was conducted in accordance with the Declaration of Helsinki and was approved by the local Ethics Committees of all participating centers. Written informed consent was obtained from all participating patients.

Safety assessments

Safety was assessed by clinical and laboratory testing, and by evaluating all adverse events reported at each visit. In accordance with the protocol, growth factors were recommended to encourage optimum patient compliance in relation to predictable hematological side effects such as anemia.

Erythropoietin (EPO; Epogin, Chugai) from 6000 IU/week was used to treat anemia (Hb levels <10 g/dl). RBV was administrated from 200 mg. When Hb increased by more than 10 g/dl, 200 mg per day of RBV was added. The daily dose of RBV was reduced by 200 mg when Hb fell below 10 g/dl, an acute decrease was followed by stabilization of Hb concentration at more than 3 g/dl from baseline, or the appearance of clinical symptoms of anemia (e.g. palpitation, dyspnea on effort, and fatigue) associated with a decrease in Hb of >2 g/dl from baseline. Once the RBV dose was reduced, it was maintained at that level throughout the rest of study if patients complained of anemia-related symptoms of fatigue or pallor. However, RBV was discontinued when Hb fell below 8.5 g/dl or when patients manifested more severe anemia, including orthostatic hypotension. PEGIFN was stopped if significant side effects occurred or if cytopenia persisted (neutrophil count <750/mm^3, platelet count <20,000/mm^3).

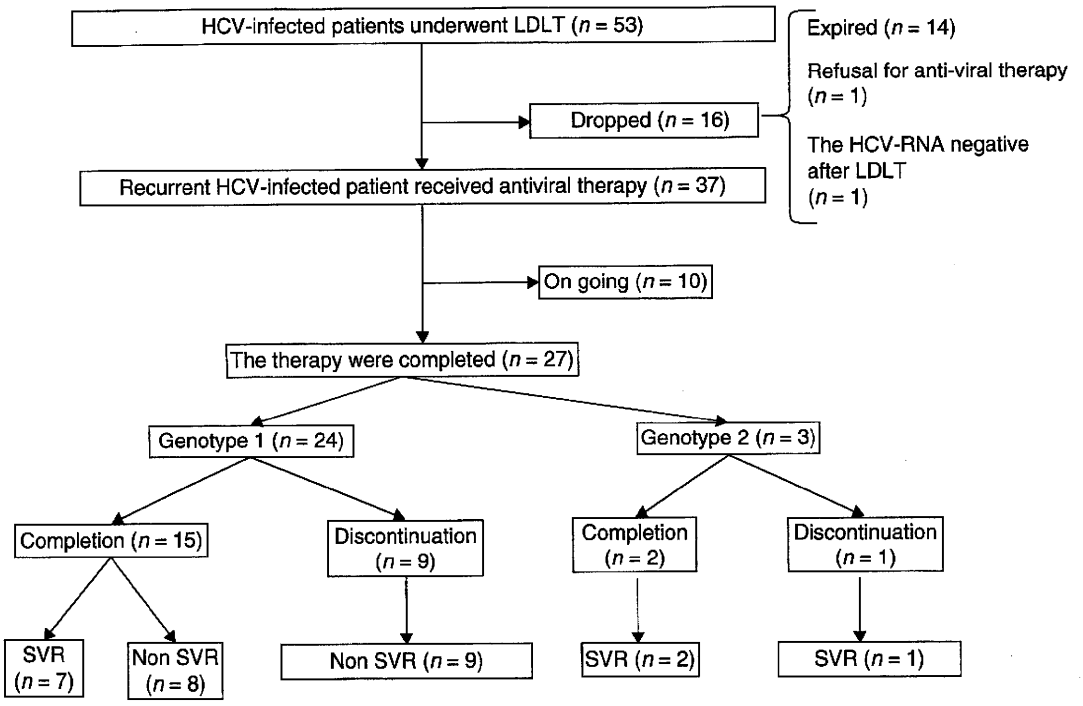

Figure 1. Flow diagram showing the course of HCV-infected patients after living donor liver transplantation. Twenty-seven patients treated with PEGIFN/RBV combination therapy were divided into two groups, namely sustained virological response (SVR) and non-SVR. *n*, number of patients. HCV genotype is shown.

Assessment of efficacy

HCV RNA levels were measured using one of several RT-PCR-based methods (original Amplicor method, high range method, or TaqMan RT-PCR test) at weeks 2 and 4, every 4 weeks of treatment thereafter, and at 24 weeks after the cessation of therapy.

The response was considered to be a SVR after another 6 months of negative serologic results without antiviral treatment. Patients with positive qualitative HCV RNA PCR tests during all examinations were categorized as having a non-virological response. Virological response (VR) was defined as becoming PCR-negative at least once during treatment; early virological response (EVR) as HCV-RNA-positive at 4 weeks after the start of treatment and HCV-RNA-negative at 12 weeks; and late virological response (LVR) as HCV-RNA-negative at more than 13 weeks after the start of treatment.

Analysis of nucleotide sequence of the core and NS5A region

Fifteen patients with genotype 1b completed our protocol. Seven patients achieved SVR whereas eight did not. Using serum obtained before LDLT, we analyzed amino acid (aa) substitutions at aa 70 and aa 91 of the HCV core region (HCV CR) and mutation at the interferon sensitivity-determining region (ISDR) in the nonstructural 5A (NS5A) region of HCV by the direct sequencing method. The core aa 61–110 and NS5A aa 2209–2248 (IFN-sensitive determining region [ISDR]) [15] sequences were determined by direct sequencing using stored serum samples obtained just before therapy. HCV RNA was extracted from serum samples and reverse transcribed with random primers and MMLV reverse transcriptase (Takara Bio Inc., Shiga, Japan). DNA fragments were amplified by PCR using the primers below. Nucleotide sequences of the core region: first-round PCR was performed with primers CC11 (forward, 5′-GCC ATA GTG GTC TGC GGA AC-3′) and e14 (reverse, 5′-GGA GCA GTC CTT CGT GAC ATG-3′), and second-round PCR with primers CC9 (forward, 5′-GCT AGC CGA GTA GTG TT-3′) and e14 (reverse), as described by Akuta et al. [16,18,21]. After denaturation at 95°C for 5 min, 35 cycles of amplification were set as follows: denaturation for 30 s at 94°C, annealing of primers for 1.5 min at 57°C, and extension for 1 min at 72°C, followed by final extension at 72°C for 7 min. The second PCR was carried out with the same amplification conditions as those used in the first PCR, except that the second PCR primers were used instead of the first PCR primers. Nucleotide sequences of ISDR in NS5A: PCR was performed

with IM11 (forward, 5′-TTC CAC TAC GTG ACG GGC AT-3′) and 5OA2KI (reverse, 5′-CCC GTC CAT GTG TAG GAC AT-3′). After denaturation at 98°C for 30 s, 35 cycles of amplification were set as follows: denaturation for 10 s at 98°C, annealing of primers for 30 s at 66°C, and extension for 15 s at 72°C, followed by final extension at 72°C for 5 min. The amplified PCR products were separated on a 2% agarose gel and purified by GENECLEAN II kit (Q-Bio Gene, Carlsbad, CA). Nucleotide sequences were determined using Big Dye Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer, Tokyo, Japan). Nucleotide and aa sequences were compared with the nucleotide sequences of genotype 1b HCV-J (Gene Bank accession number; D90208) [22].

Statistical analysis

Variables between the SVR and non-SVR groups were compared using non-parametric tests (Mann–Whitney *U* test, two-tailed test and Fisher's exact probability test). Analyses for efficacy and safety were conducted on an intention-to-treat (ITT) basis, performed on patients who received at least one dose of the study medication.

Predictors of SVR were determined using univariate analyses. All p values <0.05 by two-tailed tests were considered significant. Potential predictive factors associated with SVR included sex, age, body mass index (BMI), viremia level, number of mutations in the ISDR, HCV core region (double mutant/non-double mutant), time from transplantation to therapy, duration of treatment, adherence to PEGIFN treatment, and adherence to RBV and EVR treatment. Statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL).

Results

Patients characteristics

Table I shows the baseline characteristics of the 27 patients with recurrent hepatitis C after LT who were treated with PEGIFN/RBV combination therapy. The median age of patients was 56 years, and 17 were male. Median body mass index was 24.3. Most patients were infected with HCV genotype 1 ($n = 24$) and genotype 2 ($n = 3$). Median time for the initiation of antiviral therapy after transplantation was 4 months, and median pretreatment serum HCV RNA levels were 6.6 log IU/ml. Immunosuppressive therapy included tacrolimus in 22 of 27 patients, and cyclosporine in 5 of 27.

Table I. Characteristics of 27 patients with recurrent hepatitis C after living donor liver transplantation.

Age (years)*	56 (29–69)
Gender (male/female)	17/10
Body mass index*	24.3 (14.8–42.2)
Genotype (1/2)	24/3
Viral load at therapy (log IU/ml)*	6.6 (4.9–7.8)
Time from transplantation to therapy (months)*	4 (1–41)
Immunosuppression (tacrolimus/cyclosporine)	22/5

*Values are median (range).

Efficacy and safety assessment

Among 27 patients who were treated with antiviral therapy, 17 were able to complete our protocol (15 patients with genotype 1, 2 patients with genotype 2), whereas 10 patients had to discontinue the protocol (9 patients with genotype 1, 1 patient with genotype 2). SVR rate with PEGIFN/RBV was 37.0% (10/27). By genotype, SVR rate in patients with genotype 1 was 29.2% (7/24) and 100% (3/3) in those with genotype 2 (Figure 1). Most patients with genotype 1b whose HCV RNA reached undetectable level achieved SVR, at 87.5% (7/8), with only one patient not achieving SVR (Table II) (Figure 2).

Ten patients discontinued treatment, due to liver failure owing to the recurrence of HCV in 5 patients, general fatigue in 2, ALT flare due to acute rejection in 1 patient, anemia in 1, and depression in 1 (Figure 1).

Efficacy of long-term interferon therapy for genotype 1b patients

Table II shows details of patients who were treated with PEGIFN/RBV until HCVRNA had reached undetectable levels and were then further treated for at least more than 1 year.

Seven patients achieved SVR by prolonged PEGIFN/RBV for at least 1 year or more. Seven patients were male.

Eight patients had reached undetectable levels of HCV RNA and 7 patients had never reached undetectable levels of HCV RNA. Although 5 of the 8 patients were classified as LVR, 4 patients of these 5 achieved SVR. One male patient aged 69 years (patient no. 5) who had double mutation of aa 70 and aa 91 in the core region and zero substitutions in ISDR achieved SVR after prolongation of therapy (Figure 3). By contrast, another male aged 51 years (patient no. 9) who had double wild aa 70 and aa 91 in the core region and five substitutions in the ISDR did not achieve SVR after prolongation of therapy (Figure 4).

Table II. Details of 15 patients (genotype 1).

Patient no.	Age (years)	Gender	HCV RNA (log IU/ml)	HCV core region (aa 70/aa 91)	Number of mutations in the ISDR	Time from transplantation to therapy (months)	Time to reach undetectable levels of HCV RNA (weeks)	Treatment duration of VR (weeks)	Treatment duration (weeks)	Adherence to PEGIFN (%)	Adherence to RBV (%)	SVR
1	63	Male	6.1	m/m	4	41	3	103	106	35	29	Yes
2	60	Male	5.8	w/w	1	13	10	48	58	100	62	Yes
3	66	Male	6.6	m/m	3	12	12	60	72	80	11	Yes
4	54	Male	6.5	w/w	3	4	16	56	72	77	54	Yes
5	69	Male	6.3	m/m	0	12	21	57	78	42	14	Yes
6	44	Male	6.6	w/m	0	1	28	76	104	88	47	Yes
7	53	Male	6.1	m/w	1	2	54	125	179	57	25	Yes
8	56	Male	6.6	m/w	0	2	27	52	79	66	7	No
9	51	Male	5.9	w/w	5	6	NR	NR	173	80	25	No
10	47	Female	6.6	m/m	1	3	NR	NR	124	63	15	No
11	59	Female	6.6	m/w	1	7	NR	NR	86	100	65	No
12	64	Female	5	m/m	0	3	NR	NR	81	72	25	No
13	58	Female	6.6	m/m	1	3	NR	NR	79	83	63	No
14	65	Female	5.9	w/m	0	3	NR	NR	79	45	19	No
15	56	Male	7.2	m/w	0	3	NR	NR	58	51	26	No

Abbreviations: m = mutant; w = wild; NR = non-virological responder; VR = virological response; SVR = sustained virological response.

Predictive factors of SVR in genotype 1b patients

Among 15 patients who completed our protocol with genotype 1b, Potential predictive factors associated with SVR were analyzed. Variables were follow up, the age, gender, body mass index, duration for the initiation of antiviral therapy after transplantation, pretreatment serum HCV RNA levels, immunosuppressive therapy, the number of mutations in the ISDR, HCV core region (double mutant/non-double mutant) adherence of PEGIFN and adherence of RBV.

There was no significance difference between the SVR and non-SVR groups among the 15 patients with genotype 1b in our study (Table III). EVR rates in the SVR group tend to be higher than that of the non-SVR group, albeit that the difference was not significant ($p = 0.07$) (Table III). Mutation of aa 70 and aa 91 in the core region of the HCV protein and fewer mutations in its ISDR region did not significantly differ between the SVR and non-SVR groups among the 15 patients with genotype 1b in our study.

Although it has been reported that mutation of aa 70 and aa 91 in the core region of the HCV protein is predictive of a non-virological response [17,18], all three patients who had double mutation of aa 70 and aa 91 in the core region achieved SVR in this study.

Moreover, although it has also been reported that fewer mutations in the ISDR region of the HCV protein is predictive of a non-virological response [15], all four patients with 0 or 1 mutation in the ISDR achieved SVR.

Discussion

The optimal duration of therapy for liver transplant recipients with recurrent HCV is unclear. The treatment period for immunocompetent patients in the majority of published studies is 48 weeks. Among immunocompetent patients, the probability of relapse was greater in those responding later [23,24]. Using a mathematical model, Drusano and Preston reported that genotype 1-infected patients require the continuous absence of detectable HCV RNA in serum for 36 weeks to attain 90% probabilities of an SVR (i.e. relapse rate 10%) [25]. It is recently recommended that 72-week IFN treatment, compared to 48-week standard IFN treatment, was effective for the untransplanted patients with chronic hepatitis C whose HCV RNA does not reach undetectable level within 12 weeks [11,12]. It is also well known that patients with recurrent chronic hepatitis after LDLT are unlikely to achieve SVR, compared to immunocompetent

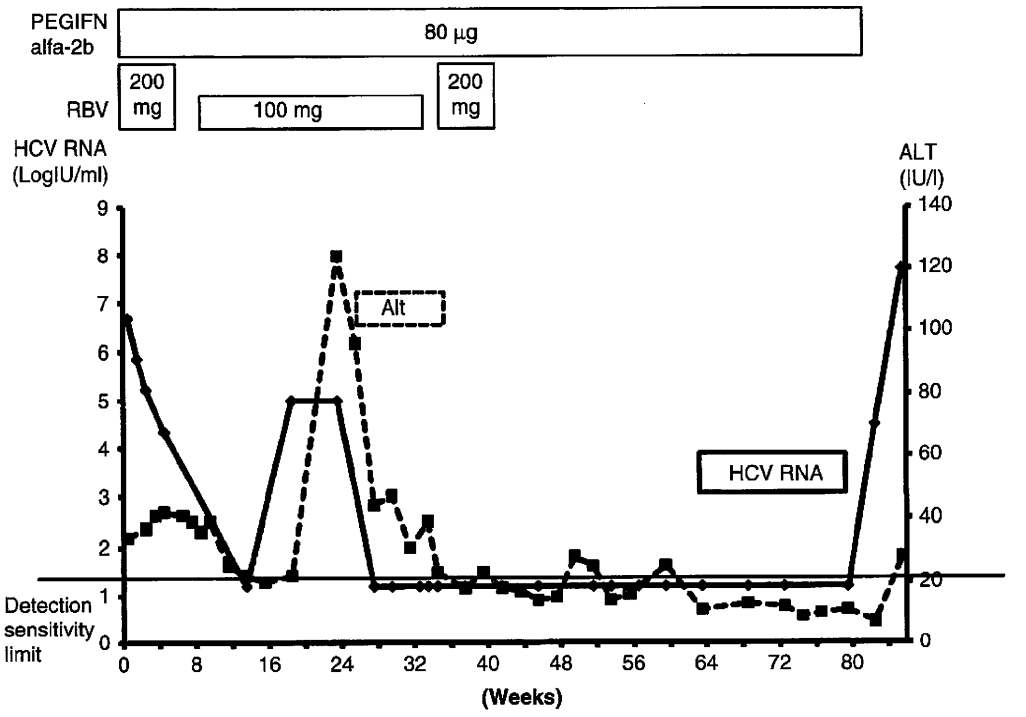

Figure 2. Clinical course in a male patient aged 56 years with genotype 1b, HCV Core 70mutant 91wild, and 0 ISDR mutations. Serum HCV RNA became negative at 27 weeks, after which treatment duration was 52 weeks. However, HCV RNA became positive at 1 week after the cessation of treatment.

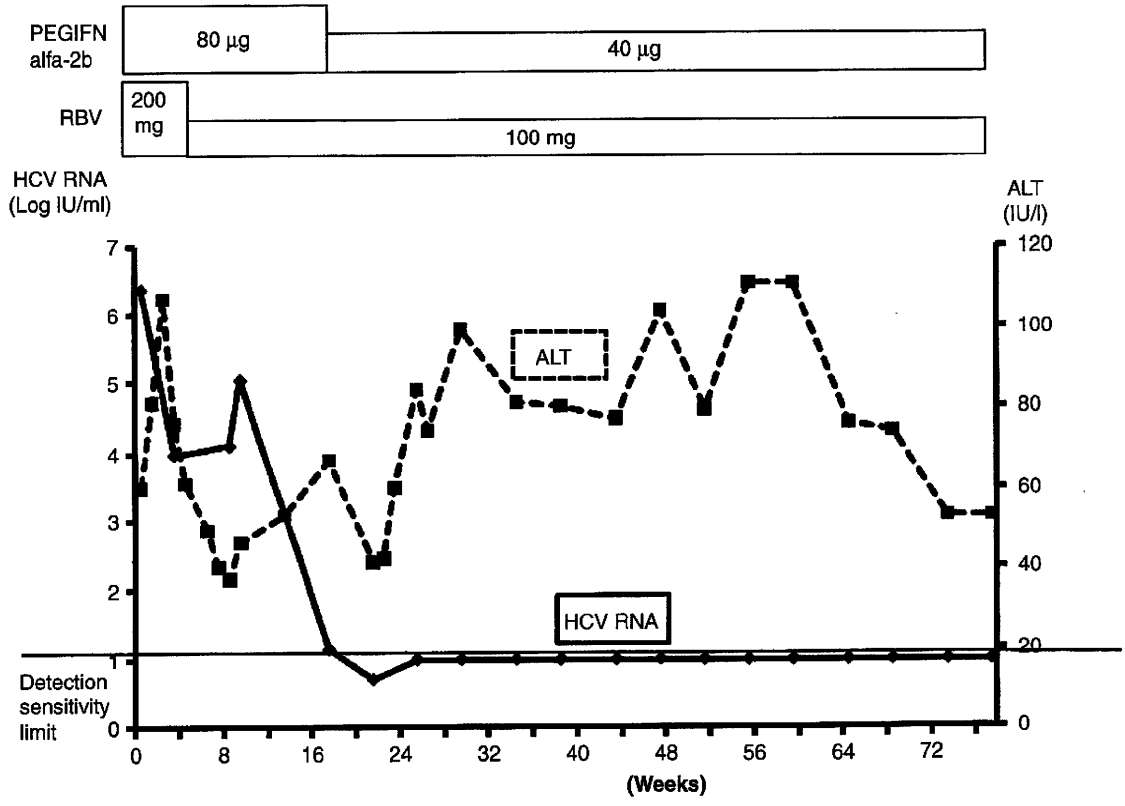

Figure 3. Clinical course in a male patient aged 69 years with genotype 1b, HCV Core 70mutant 91mutant, and 0 ISDR mutations. Virological response occurred at 21 weeks and therapy continued to 78 weeks. Final status was SVR.

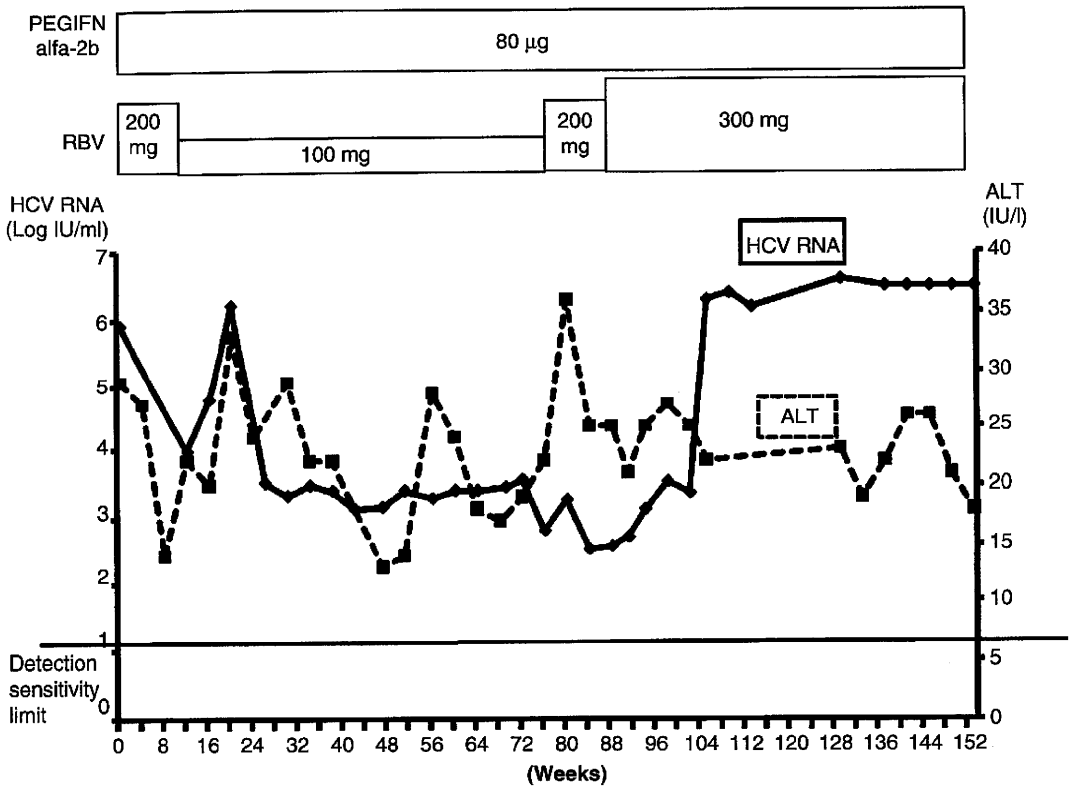

Figure 4. Clinical course in a male patient aged 51 years with genotype 1b, HCV Core 70wild 91wild, and 5 ISDR mutations. He did not develop VR during the dosing period.

untransplanted patients. Consequently, prolonged IFN treatment would be useful and improve the SVR rate for transplanted patients.

We treated recipients with PEGIFN/RBV until HCV RNA had reached undetectable levels and then to continue treatment for at least 1 year. 62.9% of patients (17/27) could complete therapy without severe adverse affects (Figure 1) and relapse rate under this study was 12.5% (Table II), whereas Tamura and Ueda reported a relapse rate of 14% and 3% under the same treatment. As a result, SVR rate was 34% and 50%, respectively [13,14]. With the aid of these results, it might indicate that prolonged PEGIFN/RBV therapy would be useful in eradicating HCV in LDLT patients.

In our study, seven patients achieved SVR by prolonged PEGIFN/RBV for at least 1 year or more. Three of seven patients were EVR and four were LVR.

By contrast, in eight patients who were non-SVR, only one patient had reached undetectable levels of HCV

Table III. Predictive factors associated with SVR in genotype 1b patients.

	SVR (n = 7)	Non-SVR (n = 8)	p-Value
Age (years)*	60 (44–69)	57 (47–65)	0.64
Gender (male/female)	7/0	3/5	0.07
Body mass index*	24.1 (21.4–26.5)	24.2 (18.9–42.2)	0.67
Viral load at therapy (log IU/ml)*	6.3 (5.8–6.6)	6.6 (5.9–7.2)	0.48
Time from transplantation to therapy (months)*	12 (1–41)	3 (3–7)	0.21
Number of mutations in the ISDR (0–1/2–5)	4/3	7/1	0.28
HCV core region (double mutant/non-double mutant)	3/4	3/5	0.6
Duration of treatment (week)*	72 (48–179)	75 (61–133)	0.7
Immunosuppression (tacrolimus/cyclosporine)	6/2	7/1	1
Adherence of PEGIFN (%)*	80 (35.5–100)	71.5 (45.4–100)	0.39
Adherence of RBV (%)*	47.4 (11.2–62.5)	25.5 (15.3–65.9)	0.74
Early virological response (yes/no)	3/4	0/8	0.07

*Values are median (range).

RNA and other seven patients had never reached undetectable levels of HCV RNA. That is, if patients had reached undetectable levels of HCV RNA, they could eradicate HCV RNA in the liver tissue by prolonged IFN therapy for more than 48 weeks after HCV RNA reached undetectable levels. This regimen is similar to that of a recent recommendation that PEGIFN/RBV therapy for 72 weeks is necessary for patients with chronic hepatitis C whose HCV RNA does not reach undetectable levels within 12 weeks.

Recent findings among immunocompetent patients of pretreatment factors that could predict treatment efficacy of 72-week PEGIFN/RBV identified substitution of either or both aa 70 or 91 in the HCV core region, and the number of substitutions in amino acids 2209–2248, the ISDR of NS5A in HCV genotype 1b [26]. By contrast, however, our present results showed that substitution of aa 70 and/or 91 in the HCV core region or the number of ISDR were not predictive of SVR (Table III). All three patients who had double mutation of aa 70 and aa 91 in core region of HCV protein achieved SVR in this study, as did all four patients whose number of mutations in the ISDR was 0 or 1 (Table II).

Recently Fukuhara et al. reported that mutations of the HCV core and NS5A regions of HCV genome were associated with the SVR rates in 50 patients [27]. Although the number of our patients included was less than Fukuhara's, we think that our result is still worth reporting because, in the case of acute hepatitis C, 24-week IFN treatment is enough to eradicate HCV in most cases, suggesting that HCV core mutant and the substitutions of amino acids of the HCV NS5A region are not likely to affect the SVR rate for acute hepatitis C. Since the recurrence of hepatitis C for transplanted patients is another acute hepatitis C, those substitutions might not affect the SVR rate of IFN treatment. Further studies would reveal whether the mutations of the HCV core and NS5A regions of HCV genome were associated with the SVR rates.

Only one patient had HCV relapse after 79 weeks treatment, a male aged 56 years with genotype 1b, HCV Core 70mutant 91wild and number of ISDR mutation 0 (Figure 2). He had a VR at 27 weeks, which lasted for 52 weeks, and continued therapy to 79 weeks. However, he subsequently experienced relapse of HCV. One of many possible reasons was likely low adherence to RBV (7%).

Several reasons may account for the lack of association of HCV core region mutation and number of ISDR mutations with SVR rate. One reason is that it is acute hepatitis after LDLT, which is usually treated as soon as possible: even in those infected with genotype 1, HCV could be eradicated with regular IFN for 24 weeks after acute infection [28–31], meaning that mutation of the core region and NS5A could not be determinants of PEGIFN therapy in LDLT cases. A second reason might be poor adherence to PEGIFN and RBV treatment in patients with LDLT. Among patients who experience severe leucocytopenia, thrombocytopenia and anemia after LDLT, dose reductions in PEGIFN or RBV are therefore inevitable. Therefore, it is reasonable to prolong the duration of PEGIFN/RBV therapy. Taken together, recurrent hepatitis C after LDLT is different from hepatitis C in immunocompetent patients. This might be the reason why any predictive factor but EVR was an only predictive factor of SVR in this study.

There were several limitations in present study. One was that our study is retrospective.

Since it was scheduled that the end point of treatment should be 1 year after serum HCV RNA became negative, it compelled to design the retrospective study as a pilot study. Further prospective study will prove our protocol strongly and help achieving high SVR and low relapse rate. Another limitation was the low number of patients included. Although other institutes also demonstrated good results with similar interferon protocol, as mentioned above [13,14], another study with more number of patients will prove the consistence of our study.

In conclusion, for recurrent hepatitis C after LDLT, our findings indicate that PEGIFN therapy for at least 1 year after HCV RNA reaches undetectable levels might prevent HCV viral relapse. Combination of the new selective inhibitors of HCV, named STAT-C (specifically targeted antiviral therapy for HCV), is expected to further improvements in SVR rates.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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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.

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), 25OAS and MxA mRNAs that mediate antiviral effects (Itsui et al., 2009; Itsui et al., 2006). Induction of 25OAS 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

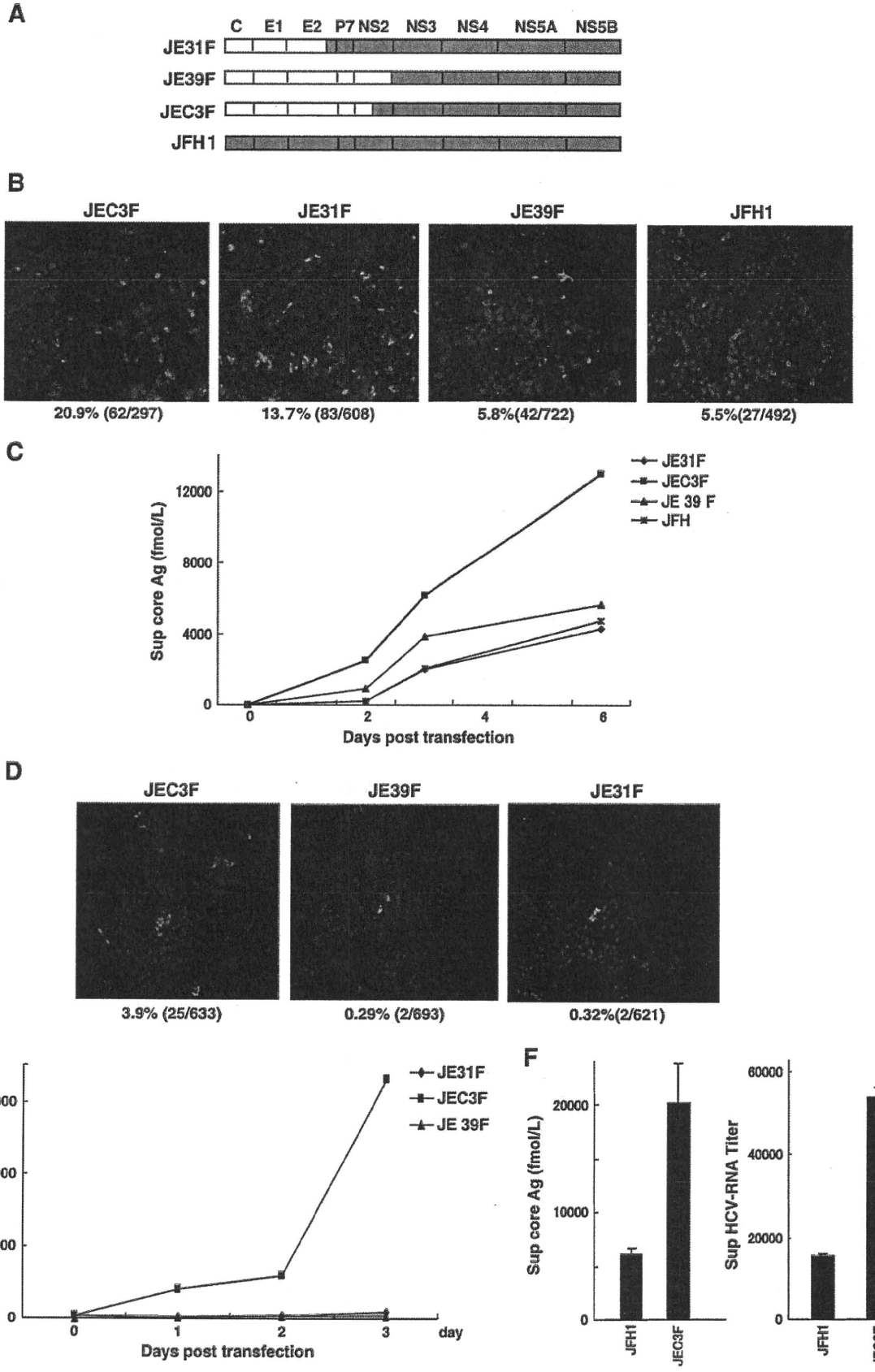
It has been reported that SOCS3 is induced principally by phosphorylated STAT3 (pSTAT3) (Hanada et al., 2003) and that interleukin-6 (IL-6) is a strong inducer of pSTAT3 via receptor-mediated Janus kinase activation in the liver (Ramadori and Christ, 1999). This background led us to investigate whether 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 Huh7.5.1cell (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


A
C E1 E2 P7 NS2 NS3 NS4 NS5A NS5B
JE31F
JE39F
JEC3F
JFH1
B
JEC3F
JE31F
JE39F
JFH1
20.9% (62/297)
13.7% (83/608)
5.8%(42/722)
5.5%(27/492)
C
JE31F
JEC3F
JE 39 F
JFH
Sup core Ag (fmol/L)
12000
8000
4000
0
0
2
4
6
Days post transfection
D
JEC3F
JE39F
JE31F
3.9% (25/633)
0.29% (2/693)
0.32%(2/621)
E
JE31F
JEC3F
JE 39F
Sup core Ag fmol/L
3000
2000
1000
0
0
1
2
3
day
Days post transfection
F
Sup core Ag (fmol/L)
20000
10000
0
JFH1
JEC3F
Sup HCV-RNA Titer
60000
40000
20000
0
JFH1
JEC3F

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-medited 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 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., 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).

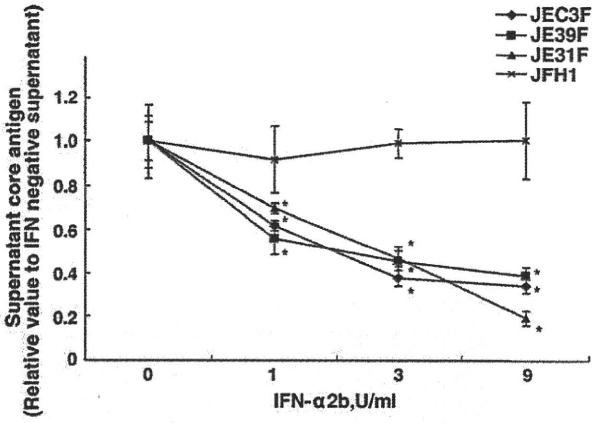


Fig. 2. Comparison IFN-alpha sensitivity among 2b/JFH1 chimeric viruses and JFH1. Ten μg of JE31F, JE39F, JEC3F, JFH1 RNA were transfected into 5×10^6 Huh7.5.1 cells. The transfected cells were divided into 12 wells. Forty eight hours after transfection, cells were washed twice with PBS and treated with 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 ± sd. Asterisks indicate p-values of less than 0.05.

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 date. 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 naïve 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 date. 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 ± sd.

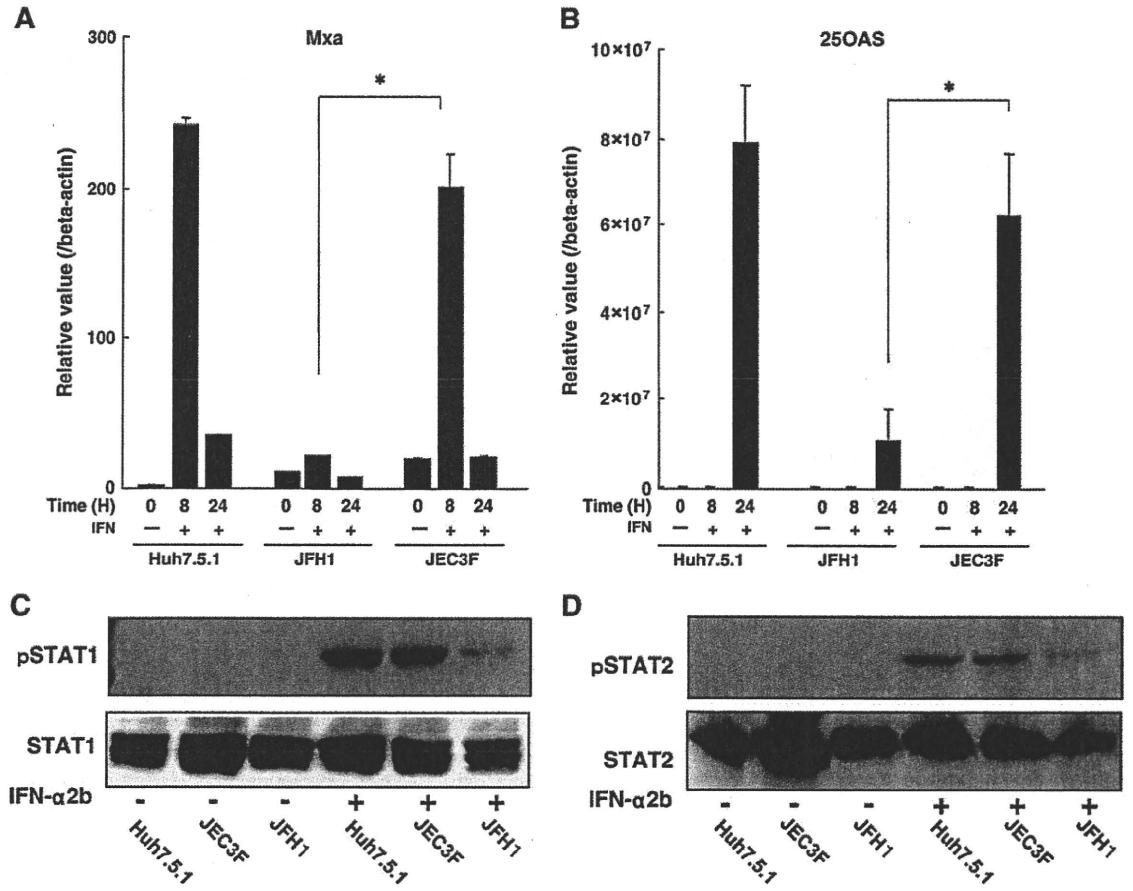


Fig. 3. Induction by interferon of the interferon-inducible genes, MxA (panel A), 25-OAS (panel B) and phosphorylated STAT1 (panel C) and STAT2 (panel D). JEC3F and JFH1 10 μg RNA was transfected into Huh7.5.1 cells. Forty-eight hours after transfection, the cells were treated with 25 U/ml IFN-alpha 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-alpha 2b. Total cellular protein was isolated before and 15 minutes after IFN treatment. Ten μg 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 ± 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

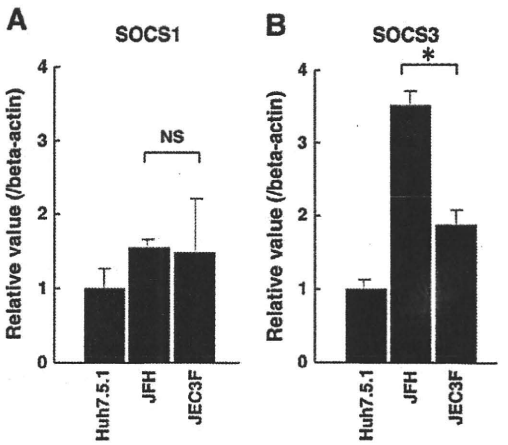


Fig. 4. Expression of SOCS1 mRNA (panel A), SOCS3 mRNA (panel B). Forty-eight hours after transfection of JEC3F, JFH1 10 μg RNA or mock transfection into Huh7.5.1 cells, total RNA and total protein were isolated. Relative gene expression levels of SOCS1 (panel A) and SOCS3 (panel B) and were determined 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 ± sd. Asterisks indicate p-values of less than 0.05.

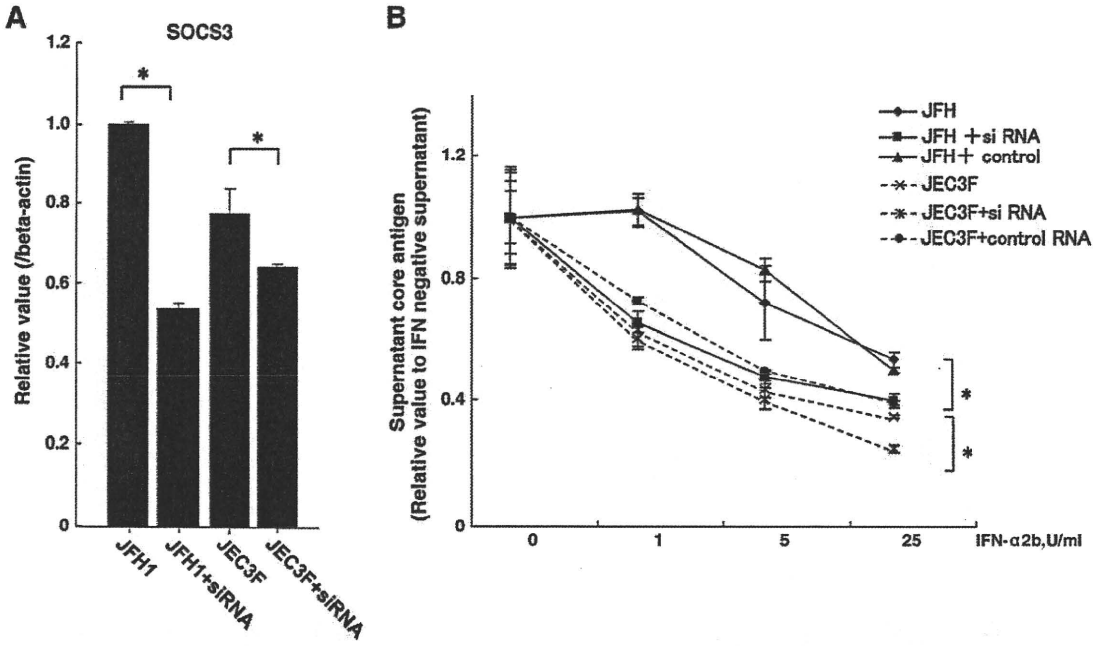


Fig. 5. Differences in sensitivities to IFN between SOCS3-knock down, HCV transfected cells. JFH1 or JEC3F 10 μg RNA, and 80 pmol siRNA SOCS3-HSS113312 or MOCK were electroporated into 5×10^6 uninfected Huh7.5.1 cells. A. Expression of SOCS3 mRNA in uninfected and HCV-infected Huh7 cells. Forty-eight 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 ± 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-alpha 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 ± 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 μl 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® 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.

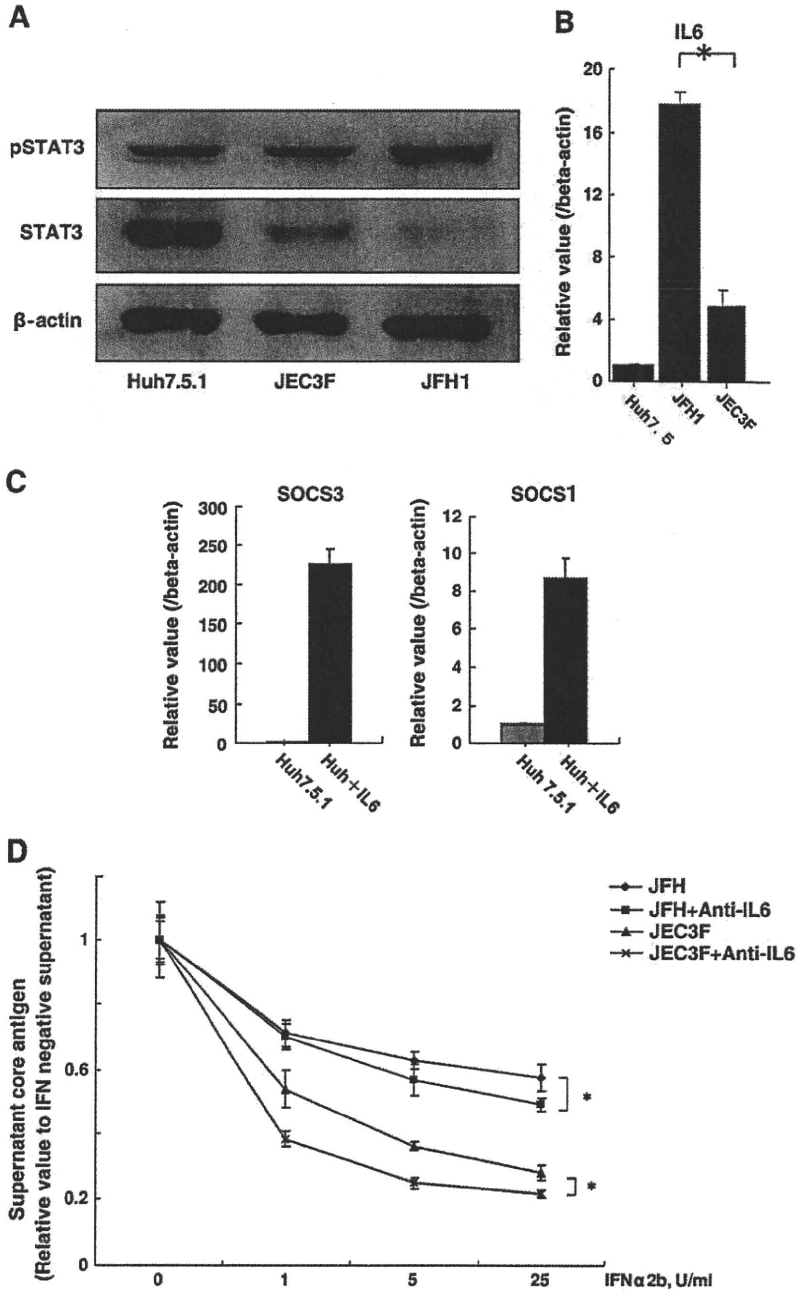


Fig. 6. IL-6 expression in HCV infected cells and change in IFN sensitivity by treatment with anti-IL6 antibody. A. Expression of cytoplasmic phospho-STAT3 in uninfected and HCV-infected Huh7 cells. JEC3F, JFH1 10 μg RNA and MOCK was transfected into Huh7.5.1 cells. Forty eight hours total cellular protein was isolated. Ten μg 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 ± 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 minuets 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 is mean ± 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 μg/ml anti-IL6 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 ± 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 ± 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 ± sd. Asterisks indicate p-values of less than 0.05.

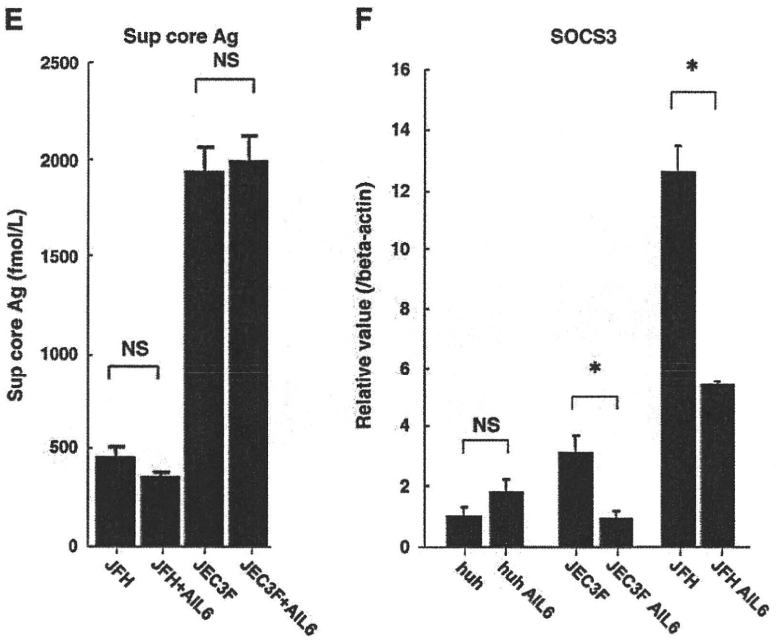


Fig. 6 (*continued*).

Construction of 2b/JFH-1 based intragenotypic chimeras and transfection

Chimeric HCV constructs of HCV-2b and JFH1 were shown in Figs. 1A and 7A. To construct 2b/JFH1-based intragenotypic chimera, JE31F, the 2b sequence of core through E2 (nt. 342–2541) was fused to the EcoRI-JFH1–5′-untranslated region (UTR) DNA by fusion PCR. The fused 5′UTR-E2 fragment and JFH1-E2-NS3 (nt2541 through 5324) were assembled by fusion PCR and cloned into pGEM-T EASY. The product was digested by EcoRI and AfeI and insert into pJFH1. Plasmids pJE39F, pJEC3F, pJcoreC3F and p2bcore JFH1 were constructed using a similar procedure. Plasmids pJEC3F and pJE39F were joined between NS2 and NS3, and within NS2 at nt. 2867, respectively. Plasmid pJcoreC3F was made by substitution of the core region of 2b/JFH1 with that of JFH1. The plasmid p2bcoreJFH1 was made by substitution of the core region of JFH1 with that of 2b/JFH1.

Cells and cell culture

Huh7.5.1 cells were maintained in Dulbecco's modified minimal essential medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum at 37 °C under 5% CO_2.

HCV cell culture system

Full-length HCV expression plasmids were as follows: pJFH1-full (Wakita et al., 2005), pJE31F, pJE39F, pJEC3F, pJcoreC3F, p2bcoreJFH1, and pFL-H77/JFH1, pFL-J6/JFH1 (Lindenbach et al., 2005). These plasmids were linearized at their 3′ ends and used as templates for HCV RNA synthesis using the RiboMax Large Scale RNA Production System (Promega, Madison, WI). After DNase I (RQ-1, RNase-free DNase, Promega) treatment, the HCV RNA was purified using ISOGEN (Nippon Gene, Tokyo, Japan). For the RNA transfection, Huh7.5.1 cells were washed twice with PBS, and 5×10^6 cells were suspended in Opti-MEM I (Invitrogen Carlsbad, CA) containing 10 μg of HCV RNA, transferred into a 4 mm electroporation cuvette and finally subjected to an electric pulse (1,050 μF and 270 V) using the Easy Jet system (EquiBio, Middlesex, UK). After electroporation, the cell suspension was left for 5 min at room temperature and then incubated under normal culture conditions in a cell culture dish.

Quantification of HCV core antigen in culture supernatants

Culture supernatants of HCV RNA transfected Huh7.5.1 cells were collected on the days indicated, passed through a 0.45 μm filter (MILLEX-HA, Millipore, Bedford, MA) and stored at -80 °C. The concentrations of core antigen in the culture supernatants were measured using a chemiluminescence enzyme immunoassay (CLEIA) according to the manufacturer's protocol (Lumipulse Ortho HCV Antigen, Ortho-Clinical Diagnostics, Tokyo, Japan).

Re-infection analyses

Titer-adjusted supernatants (including 0.03 fmol HCV core antigen) from HCV RNA-transfected cells were inoculated onto naïve Huh7.5.1 cells plated on a 6 cm plate at a density of 3×10^5 cells per plate. Forty-eight hours after inoculation, anti-core immunostaining was carried out with mouse anti-HCV core protein monoclonal antibody and the numbers of infected cells were counted. HCV core antigen in culture supernatants was measured at 24 hours, 48 hours, 72 hours and 144 hours after inoculation.

Real-time RT-PCR analysis

For the detection of HCV RNA in culture supernatant, supernatant was passed through a 0.45 μm filter (MILLEX-HA, Millipore, Bedford, MA) and stored at -80 °C until use. Protocol and primers for the realtime RT-PCR analysis of HCV-RNA has been described previously (Sekine-Osajima et al., 2008). For the detection of endogenous mRNAs, total cellular RNA was isolated using ISOGEN (Nippon Gene). Two micrograms of total cellular RNA were used to generate cDNA from each sample using SuperScript II. Expression of mRNA was quantified using the TaqMan Universal PCR Master Mix and the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City CA). Some primers have been described (Sekine-Osajima et al., 2008). SOCS3; forward, 5′-CAC ATG GCA CAA GCA CAA GAA G-3′ and reverse,

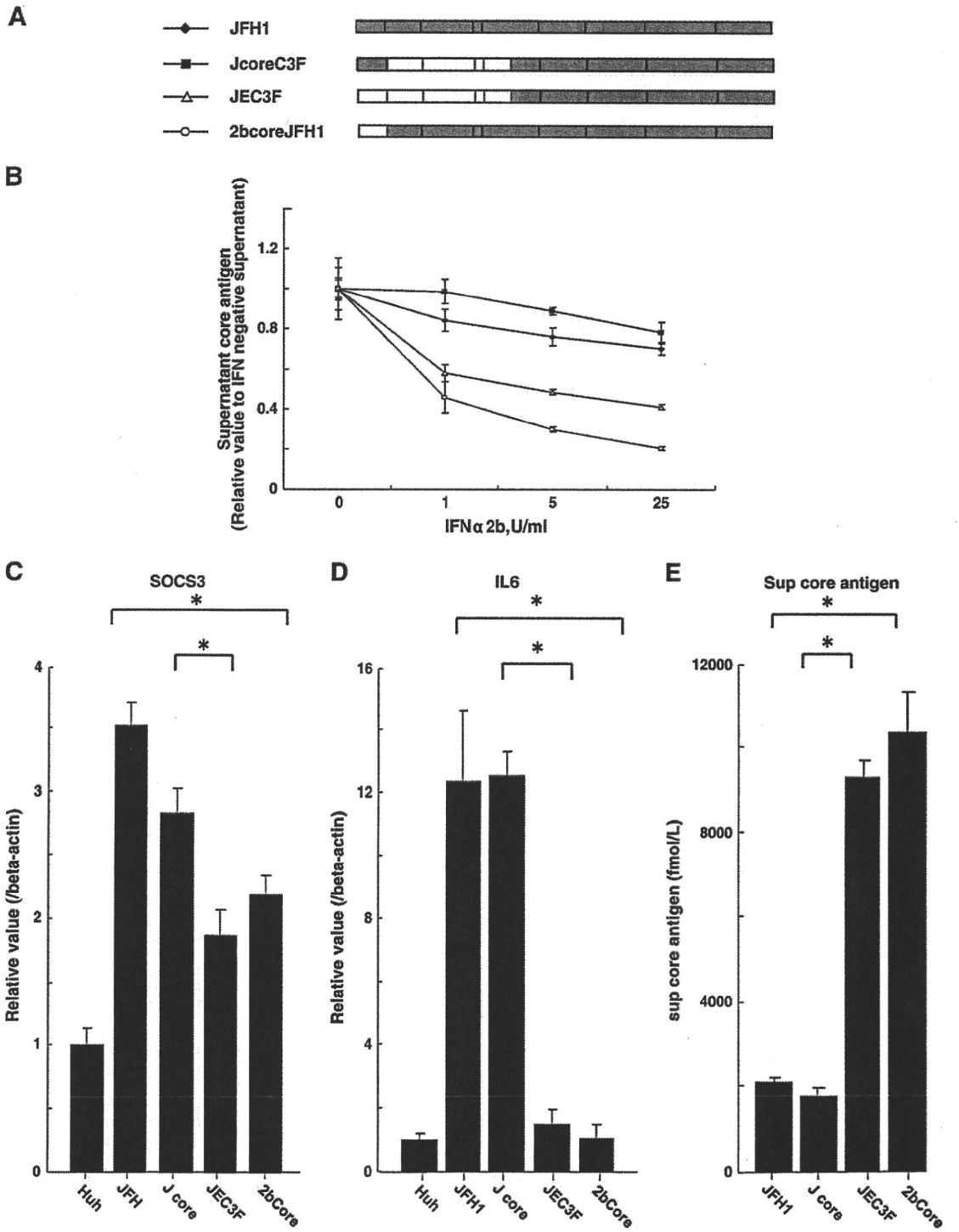


Fig. 7. Replacement of the HCV-2b-core region with JFH1-core causes upregulation of SOCS3 and IL-6 and restores resistance to IFN. A. Genome maps of JFH-1, JEC3F , J core C3F, 2b core JFH1 recombinant cDNA. J core C3 F was made by substitution of the core region of 2b/JFH1 with that of JFH1. The 2b core JFH was made by substitution of the core region of JFH1 with that of 2b/JFH1. B. Comparison of IFN-alpha sensitivity among JFH1 and JEC3F and core region substitution chimeric viruses. Ten μg of J core C3F, 2b core JFH1, JEC3F, JFH1 RNA were transfected into 5×10^6 Huh7.5.1 cells and were divided into 12 wells. Forty eight hours after transfection, the cells were 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 ± sd. Asterisks indicate p-values of less than 0.05. C, D. Core substitution leads to SOCS3 and IL-6 mRNA over-expression. Forty eight hours after transfection into cells, total RNA was isolated. Relative gene expression level SOCS3 (panel C) and IL6 (panel D) 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 ± sd. Asterisks indicate p-values of less than 0.05. E. Change of secretion of core protein following core protein substitution. 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 ± sd. Asterisks indicate p-values of less than 0.05.

5′-GGA GAA GCT GGA GAC TCA GGT G-3′, SOCS1; forward, 5′-CAC TTC CGC ACA TTC CGT TCG-3′ and reverse, 5′-GAG GCC ATC TTC ACG CTA AGG-3′, IL6; forward, 5′-GGT ACA TCC TCG ACG GCA TCT-3′ and reverse, 5′-GTG CCT CTT TGC TGC TTT CAC-3′, 25OAS; forward, 5′- CCA CCT TGG AAA GTG CCG ACA ATG CAG ACA-3′ and reverse, 5′-CGA GTC TTT AAA AGC GAT TGC CAG ATG ATC -3′, MxA; forward, 5′-GCC AGC AGC TTC AGA AGG CCA TGC TGC AGC -3′ and reverse, 5′-GGG CAA GCC GGC GCC GAG CCT GCG TCA GCC -3′.

The siRNAs

The siRNAs directed against SOCS3 were designed as follows: SOCS3-HSS113312 stealth (sequence 5′- CCC AGA AGA GCC UAU UAC AUC UAC U-3 ′and 5′-AGU AGA UGU AAU AGG CUC UUC UGG G-3′, Invitrogen) was used. 10 μg in vitro-synthesized HCV-RNA and 80 pmol siRNA SOCS3-HSS113312 or MOCK or control siRNA (negative universal control Med #2, Invitrogen) were electroporated into 5×10 naïve Huh7.5.1 cells using the protocol described in *HCV cell culture system*. Forty-eight hours after transfection, expression levels of SOCS3 mRNA were measured by real-time PCR. The difference in IFN sensitivity between SOCS3 knock down HCV infected cells and control HCV infected cells was determined by measuring supernatants HCV core antigen 72 hours after addition of IFN.

Immunohistochemistry for HCV core

HCV-JFH1 transfected or infected Huh7.5.1 cells were cultured on 22 mm-round micro cover glasses (Matsunami, Tokyo, Japan). For detection of HCV core, cells were fixed with cold acetone for 15 min. The cells were incubated with the primary antibodies for 1 hour at 37 °C, and with Alexa Fluor 488 goat anti-mouse IgG antibody (Molecular Probes, Eugene, OR) for 1 hour at room temperature. Cells were mounted with VECTA SHIELD Mounting Medium and DAPI (Vector Laboratories, Burlingame, CA) and visualized by fluorescence microscopy (BZ-8000, KEYENCE, Osaka, Japan).

Western blot analysis

Western blotting was performed as described (Tanabe et al., 2004). Briefly, 10 μg of total cell lysate was separated by SDS-PAGE, and blotted onto a polyvinylidene fluoride (PVDF) membrane. The membrane was incubated with the primary antibodies followed by a peroxidase-labeled anti IgG antibody, and was visualized by chemiluminescence using the ECL Western Blotting Analysis System (Amersham Biosciences, Buckinghamshire, UK).

Statistical analyses

Statistical analyses were performed using Student's *t*-test; p-values of less than 0.05 were considered statistically significant.

Acknowledgments

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Appendix A. Supplementary data

Supplementary Fig. 1. Infectivity of the full-length 2b HCV RNA and 2b/JFH1 chimeric virus, JEC3F. A. Challenge of human liver-engrafted albumin-uPA/SCID mice with culture fluid from JFH1 and JEC3F cells. Cell culture fluids from the JFH1 clone and JEC3F were injected intravenously into human liver engrafted albumin-uPA/SCID mice. Serum samples were obtained from the mice every 2 weeks after injection and the HCV RNA titer was determined. B. Fig. 1B Challenge of human liver-engrafted albumin-uPA/SCID mice by intrahepatic injection of in vitro synthesized, full-length 2b HCV RNA. Five hundred μl of RNA solution containing 30 μg of in vitro synthesized full-length 2b HCV RNA was injected into the livers of anesthetized chimeric mice through a small abdominal incision. Serum samples were obtained from the mice every 2 weeks after injection and the HCV RNA titer was determined.

Supplementary Fig. 2. Comparisons of replication efficiency of JFH1and J6/JFH1, 2b/JFH1 chimeras after transfection into Huh7.5.1-cells. A. Structures of theJ6/JFH1 and 2b/JFH1 genomes. J6 is joined between NS2 and NS3 with JFH1. 2b-HCV is joined with JFH1within NS2 at nt. 2867. B Measurements of core protein in cell culture fluids. Ten μg of JFH1, J6/JFH1, 2b/JFH1 RNA were transfected into 5×10^6 Huh7.5.1 cells and the cells were cultured in 100 mm-diameter plates. The culture fluids from JFH1, J6/JFH1, H77/JFH1or 2b/JFH1-transfected Huh7.5.1 cells were collected separately on the days indicated and the levels of core antigen were measured. These experiments were done three times with similar results independently. *Panel B* shows representative date.

Supplementary Fig. 3. Inhibition of infection by blocking CD81. Huh 7.5.1 cells were plated into a 6 well plate at 1.4×10^5 cells per well. After 48 hours, the cells were incubated with anti-CD81 or isotypematched control antibody at the concentration indicated for 1 hour. Subsequently, cells were infected with 1 ml of JEC3F stock cell culture fluids at day 2 for 4 hours and washed with PBS. 48 hours after inoculation, anti-core immunostaining was performed with mouse anti-HCV core protein monoclonal antibody (Panels B and C). Quantification of HCV core antigen was carried out in culture fluids at 48 hours after infection (Panel A).

Supplementary Fig. 4. Comparison between 2b and JFH-1 core amino acid sequence.

Note: Supplementary materials related to this article can be found online at doi:10.1016/j.virol.2010.07.041.

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