whether they correlated with clinical parameters, such as age, serum ALT, HCV-RNA, and HBV-DNA titers. No correlation was found between any of these markers and TLR2, TLR3, TLR4, or RIG-I expressions (data not shown). Therefore, the degree of expression of these sensors is not involved in the control of virus replication or liver inflammation. Their expressions in myeloid dendritic cells cultured with and without various reagents were compared. The ratio of the quantity was determined between samples with and without treatments and their positive induction was defined as more than 2.0. The kinetics of agonist-induced TLR2, TLR3, TLR4, or RIG-I expression were preliminarily examined in myeloid dendritic cells recovered from volunteers or patients. It was found that they showed a peak at 2 hr after the stimulation, which were the same either they were HCV-infected or not (data not shown). Thus, in the following experiments, cells were obtained at this point and subsequently analyzed transcripts of target genes.

In the present study, IFN-α significantly enhanced RIG-I expression in myeloid dendritic cells (Fig. 3A). A similar effect of IFN-α was observed in TLR3 and TLR4 expression, although at much lesser degrees than those of RIG-I. In chronic hepatitis C patients, serum levels of IL-6, TNF-α, or IL-10 have been reported to be higher than those in uninfected individuals, suggesting their roles in the pathogenesis of HCV infection [Spanakis et al., 2002]. However, the addition of these cytokines or IL-12 to myeloid dendritic cell did not influence TLR or RIG-I expression (Fig. 3B). As for TLR agonists, polyI:C or LPS significantly enhanced RIG-I expression, but only slightly enhanced TLR4 (Fig. 3B). TLR2 agonist Pam_3CSK_4 did not influence the levels of TLR and RIG-I (Fig. 3B). None of the HCV proteins had a positive impact on TLR2, TLR3, TLR4, and RIG-I expressions (Fig. 3B).

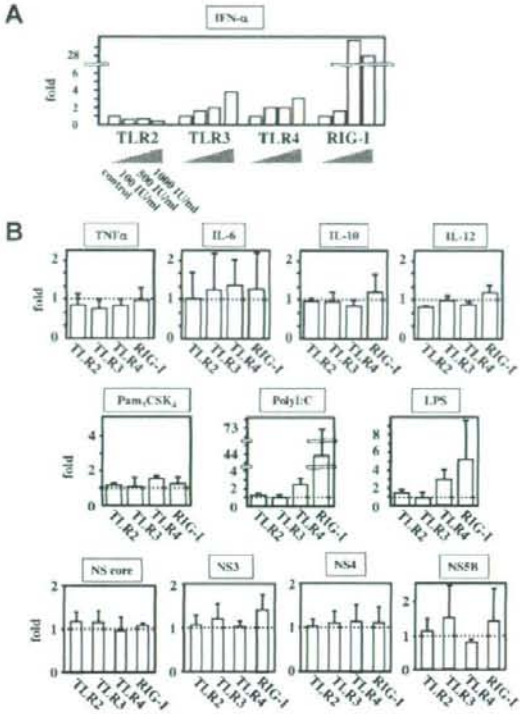

Fig. 3. IFN-α and polyI:C are inducers of TLR3, TLR4, or RIG-I in myeloid dendritic cells. **A**: Various doses of IFN-α were added to myeloid dendritic cells obtained from healthy donors and their mRNA expressions of TLR2, TLR3, TLR4, and RIG-I were quantified by real-time RT-PCR as described in Materials and Methods Section. Bars represent the mean fold increase of relevant transcripts to those of each control. Representative results from three donors are shown. **B**: Changes of TLR2, TLR3, TLR4, and RIG-I expression in myeloid dendritic cells were examined by the addition of various cytokines, TLR agonists or recombinant HCV proteins as described in Materials and Methods Section. The fold increase was determined by the ratio of each transcript of samples with reagents to those without and expressed as the mean + SEM. The concentration of reagents were 10 ng/ml of TNF-α or IL-6, 20 ng/ml of IL-10, 200 pg/ml of IL-12, 100 ng/ml of Pam3CSK4, 25 μg/ml of polyI:C, 100 ng/ml of LPS and 2.5 μg/ml each of HCV core, NS3, NS4, and NS5B. Representative results from five donors are shown.

Induction of IFN-β, TNF-α, and IL-12 p70 With TLR Agonists Is Impaired in Myeloid Dendritic Cells From Chronic Hepatitis C Patients

First, IFN-β and TNF-α expression were examined in myeloid dendritic cells as representatives in response to specific agonists. Since the expression of these genes in myeloid dendritic cell showed a peak at 2 hr after the stimulation either they were from donors or patients (Fig. 4A), samples were collected at this point. In myeloid dendritic cells stimulated with polyI:C, IFN-β was significantly induced in the HCV, the HBV, and healthy donor groups (Fig. 4B). However, their expression from HCV or HBV-infected patients was significantly lower than that from healthy donors (Fig. 4B). Agonists for TLR3 or TLR4 significantly stimulated myeloid dendritic cells to induce TNF-α regardless of HCV or HBV infection. As the same IFN-β, TNF-α induction in myeloid dendritic cells stimulated with polyI:C or LPS was lower in the HCV or the HBV group (Fig. 4B). Therefore, in myeloid dendritic cells from hepatitis C patients, in spite of higher expression of TLR2, TLR4, and RIG-I, their levels of agonist-induced IFN-β and TNF-α were less than those in healthy donors.

To compare more precisely the cytokine response in myeloid dendritic cell between HCV-infected patients and donors, the levels of IFN-α, TNF-α, IL-6, and IL-12 p70 in supernatants were examined. Since the induction of IFN-β and TNF-α in myeloid dendritic cell was profound in the presence of polyI:C, samples were collected from myeloid dendritic cells stimulated with polyI:C. The levels of IFN-α and IL-6 were not different between the groups (Fig. 4C). In contrast, the amounts of TNF-α and IL-12 p70 from patients group were significantly lower than those from the donor group (Fig. 4C). These results suggest that some inhibitory

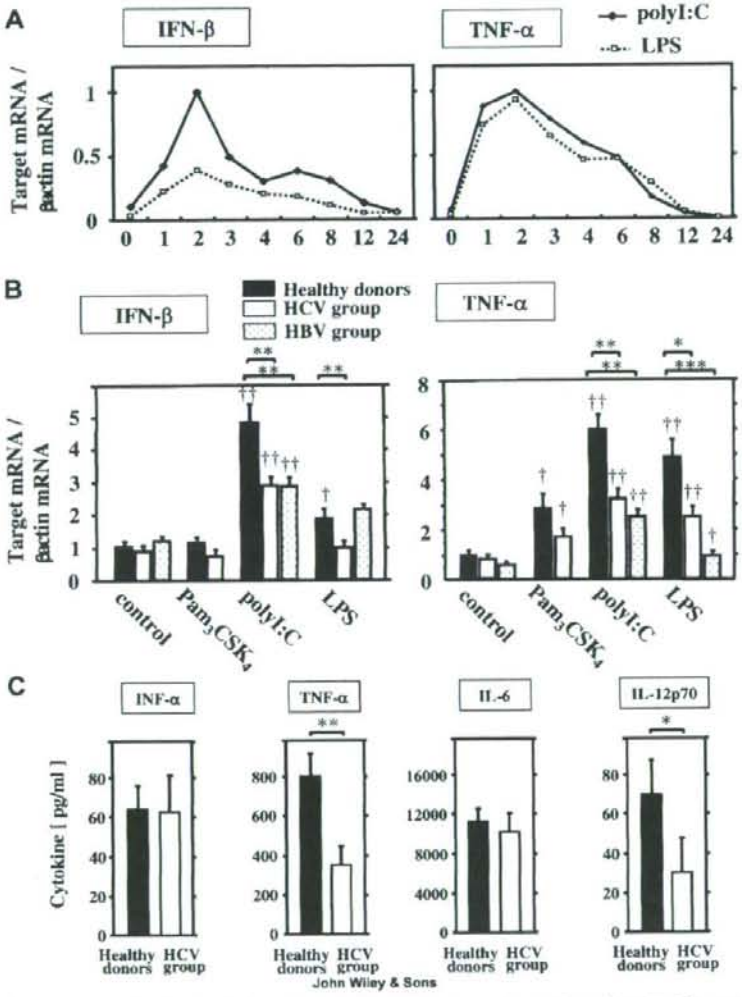

Fig. 4. Innate cytokine response is impaired in patient myeloid dendritic cells from HCV-infected patients. **A**: Kinetics of IFN-β and TNF-α in myeloid dendritic cells stimulated with polyI:C or LPS. The expressions of IFN-β and TNF-α in myeloid dendritic cells from healthy donors were quantified by real-time RT-PCR as described in Materials and Methods Section. At several time points before and after the stimulation of myeloid dendritic cell with 25 μg/ml of poly I:C or 100 ng/ml of LPS, the samples were subjected to RT-PCR analyses. The results are expressed as the ratio of IFN-β or TNF-α transcripts to that of β-actin. Representative results from three healthy donors are shown. **B**: Expressions of IFN-β and TNF-α in myeloid dendritic cells stimulated with various TLR agonists were quantified by real-time RT-PCR as described in Materials and Methods Section. Two hours after the stimulation of myeloid dendritic cells with Pam_3CSK_4, polyI:C or LPS, the samples were subjected to RT-PCR analyses. The results were expressed as the ratio of IFN-β or TNF-α transcripts to that of β-actin. The concentrations of agonists were 100 ng/ml of Pam_3CSK_4, 25 μg/ml of polyI:C and 100 ng/ml of LPS. The bars represent mean + SEM. $^{\dagger}P < 0.05$ vs. control, $^{\dagger\dagger}P < 0.01$ versus control, $^{*}P < 0.05$ versus healthy donors, $^{**}P < 0.01$ versus healthy donors, $^{***}P < 0.001$ versus healthy donors. Representative results from 14 HCV-infected patients, 13 HBV-infected patients and 25 controls are shown. Statistical differences were evaluated by the Mann–Whitney *U*-test. **C**: Myeloid dendritic cells in both groups were stimulated with polyI:C for 24 hr. The supernatants were collected and the levels of IFN-α, TNF-α, IL-6, and IL-12p70 were examined by ELISA or cytokine beads assay as described in Materials and Methods Section. The bars represent mean + SEM. Statistical differences were evaluated by the Mann–Whitney *U*-test. Representative results from 11 HCV-infected patients and 17 controls are shown. $^{*}P < 0.05$, $^{**}P < 0.01$.

mechanisms exist downstream of TLR or RIG-I in myeloid dendritic cells from the HCV-infected patients.

Expressions of TRIF and TRAF6 Were Lower in Myeloid Dendritic Cells From the HCV-Infected Patients

In order to seek the inhibitory mechanisms of TLR or RIG-I signaling in myeloid dendritic cells, the expressions of adapter molecules, MyD88, IPS-1, TRIF, or TRAF6 were compared between the HCV and donor groups. The expressions of MyD88 and IPS-1 were higher in myeloid dendritic cells from the HCV group (Fig. 5). By contrast, the levels of TRIF and TRAF6 in myeloid dendritic cells from HCV-infected patients were significantly lower than in those from healthy counterparts (Fig. 5).

DISCUSSION

The present study demonstrated that myeloid dendritic cells from HCV-infected patients express higher levels of TLR2, TLR4, and RIG-I than those from healthy subjects. Regardless of such enhanced expression, specific agonists stimulated patient myeloid dendritic cells to induce lesser degrees of IFN-β/TNF-α/IL-12 than those from the healthy counterparts. Two conclusions were reached from the current study findings: HCV enhances expression of some TLR and RIG-I in myeloid dendritic cells, but HCV impedes TLR or RIG-I-mediated cytokine responses in them. Since dendritic cells play a role as immune sentinels, such impaired cytokine response in myeloid dendritic cell may be one of the mechanisms in enhanced susceptibility to various pathogens in HCV-infected individuals as reported elsewhere [El-Serag et al., 2003].

It has been reported that TLRs are expressed in epithelial cells and immune cells, and RIG-I is ubiquitously expressed in various cells [Yoneyama et al., 2004]. However, it remains obscure how their expressions are regulated. It is generally accepted that TLR3 and RIG-I are inducible by type-I IFN [Doyle et al., 2003; Yoneyama et al., 2004]. The current study confirmed this phenomenon also in myeloid dendritic cells, since IFN-α up-regulated TLR3, TLR4, and RIG-I expression in a dose-dependent manner. Gene expression analyses revealed that HCV infection induces type-I IFN and IFN-stimulated genes in HCV-infected liver from chimpanzees or humans [Bigger et al., 2004]. One of the triggers leading to IFN production is the presence of double-strand RNA in infected tissues, which is a replicative intermediate of HCV. The current study also showed that polyI:C is a prominent inducer of RIG-I and TLR4. Since polyI:C is a synthetic mimic of double-strand RNA, its positive impact suggests that HCV replication in myeloid dendritic cells and/or subsequent IFN production may be involved in RIG-I or TLR4 induction.

Several investigators have reported that TLR2, TLR3, or TLR4 expression is enhanced in monocytes or B cells obtained from chronic hepatitis C patients, both of which are known to be susceptible to HCV [Machida et al., 2006; Riordan et al., 2006]. Regardless of the difference in cell types, the present study offers support for the enhanced TLR2 and TLR4 expression in HCV infection described by these reports. As for the mechanisms, TNF-α or HCV NS5A has been reported to be involved in TLR2 or TLR4 up-regulation [Machida et al., 2006]. However, in this study, addition of recombinant TNF-α or the HCV proteins failed to induce any TLR or RIG-I in

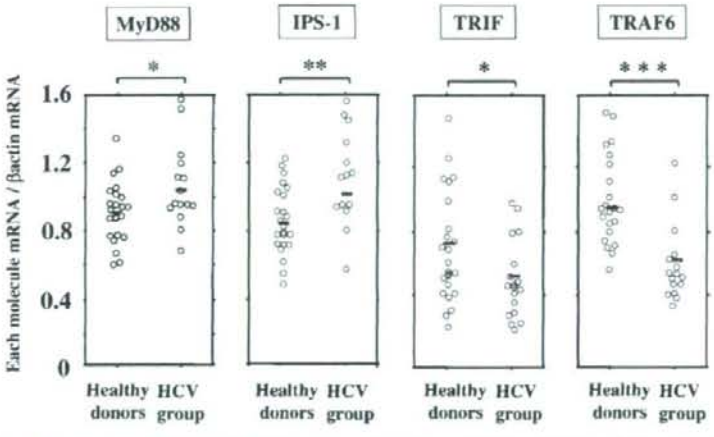

Fig. 5. Expressions of TRIF and TRAF6 are lower but those of MyD88, IPS-1 are higher in patient myeloid dendritic cells than those from healthy counterparts. Expressions of MyD88, IPS-1, TRIF TRAF6 were quantified by real-time RT-PCR as described in Materials and Methods Section. The results were expressed as the ratio of each transcript to those of β-actin. Horizontal bars represent the median. Statistical differences were evaluated by the Mann–Whitney U-test. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

myeloid dendritic cells. Therefore, enhanced expressions of TLR2, TLR4, and RIG-I in myeloid dendritic cells may be due to, not completely but in some part, the existence of HCV in cells or the exposure to endogenous IFN-α. To check this, it may be necessary to conduct studies with inoculation of HCV particles or transduction of the viral genome in myeloid dendritic cells.

In comparison of the results between the HCV and the HBV groups, the expressions of TLR2 and TLR4 in the HBV group were comparable with those from healthy donor group, suggesting that the induction of TLR2 and TLR4 in myeloid dendritic cells is unique in HCV infection. In contrast, the levels of RIG-I and LGP2 were comparable between the HCV and the HBV groups, both of which were higher than those from healthy donors. These results raise the possibility that, regardless of the difference of hepatitis virus, similar mechanisms may be involved in the induction of RIG-I and LGP2 in myeloid dendritic cells.In cells bearing HCV replicons, it has been reported that HCV NS3/4A inhibits TLR3 or RIG-I-mediated IFN-β induction by the cleavage of relevant adaptor molecules TRIF or IPS-1, respectively [Foy et al., 2005; Li et al., 2005]. In the present study, in myeloid dendritic cells from the HCV group, polyI:C-stimulated IFN-β, TNF-α, and IL-12 p70 induction is impaired. As for the adaptor molecules in TLR-dependent signals, TRIF and TRAF6 expression was lower in HCV-infected patients than those in healthy donors. Since it has been proven that the cleavage of TRIF hampers TLR3-mediated IFN production [Fitzgerald et al., 2003], the current study implies that lower expression of TRIF is involved in the inhibition of TLR3 or TLR4-mediated signals in myeloid dendritic cells. Of particular interest is the possibility that such reduction of TRIF and TRAF6 in myeloid dendritic cells is caused by the cleavage by NS3/4A, as shown in hepatoma cells [Foy et al., 2005; Li et al., 2005]. If this does occur, the inhibitor of NS3/4A serine protease may be able to restore TLR-dependent innate responses in myeloid dendritic cells, in addition to its potent suppressive ability of HCV replication. Machida et al. reported that enhanced expression of TLR4 in HCV-infected B cells is related to the TLR4-dependent up-regulation of IFN-β and IL-6, suggesting that TLR4-dependent signals are not impaired in B cells [Machida et al., 2006]. Further study is necessary to reveal whether HCV does actually influence innate immunity according to differences in blood cell types. In the current study, polyI:C or LPS-stimulated myeloid dendritic cells from HBV-infected patients induced lesser degree of IFN-β or TNF-α, respectively. Several investigators reported that the function of blood dendritic cells in HBV-infected patients were impaired [Tavakoli et al., 2004; van der Molen et al., 2004]. It is yet to be determined whether HBV infects to myeloid dendritic cells or not. The current study raises the possibility that distinct mechanisms are involved in the impairment of TLR or RIG-I pathway according to the difference of virus. Further study depending on expression as well as functional assay of virus recognition system in HBV infection is needed to clarify these important issues.

In contrast with RIG-I and LGP2, MDA-5 expression in myeloid dendritic cells from HCV-infected patients was comparable with that from healthy donors, suggesting that these cytosolic RNA sensors are regulated independently. Recently, it has been reported that RIG-I is expected to be involved in the detection of Flaviviridae, which HCV belong to, but MDA-5 is not [Hornung et al., 2006]. Active involvement of RIG-I in HCV infection has been reported, demonstrating that RIG-I, but not MDA-5, efficiently binds to secondary structured HCV RNA to confer induction of IFN-β [Saito et al., 2007]. In this study, although the polyI:C-stimulated cytokine response in patient myeloid dendritic cells was impeded, IPS-1 expression was higher than that in myeloid dendritic cells from the healthy donor group, suggesting a lesser possibility of IPS-1 as a cleavage target of HCV in myeloid dendritic cells. Alternatively, higher expression of LGP2 may contribute to the inhibitory machinery against RIG-I-mediated responses in myeloid dendritic cells, as reported elsewhere [Saito et al., 2007].

In summary, in myeloid dendritic cells from HCV-infected patients, innate cytokine responses were impaired regardless of the enhanced expressions of TLR2, TLR4, and RIG-I. These findings provide insights into the roles of the TLR/RIG-I system in the pathogenesis of HCV infection and their potentials as therapeutic targets for immune modulation.

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Short Communication

Virological characterization of the hepatitis C virus JFH-1 strain in lymphocytic cell lines

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While hepatocytes are the major site of hepatitis C virus (HCV) infection, a number of studies have suggested that HCV can replicate in lymphocytes. However, *in vitro* culture systems to investigate replication of HCV in lymphocytic cells are severely limited. Robust HCV culture systems have been established using the HCV JFH-1 strain and Huh-7 cells. To gain more insights into the tissue tropism of HCV, we investigated the infection, replication, internal ribosome entry site (IRES)-dependent translation and polyprotein processing of the HCV JFH-1 strain in nine lymphocytic cell lines. HCV JFH-1 failed to infect lymphocytes and replicate, but exhibited efficient polyprotein processing and IRES-dependent translation in lymphocytes as well as in Huh-7 cells. Our results suggest that lymphocytic cells can support HCV JFH-1 translation and polyprotein processing, but may lack some host factors essential for HCV JFH-1 infection and replication.

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Hepatitis C virus (HCV) is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (Choo *et al.*, 1989; Saito *et al.*, 1990). Infection with HCV is frequently associated with B-cell-related diseases, such as mixed cryoglobulinaemia and non-Hodgkin's lymphoma (Hausfater *et al.*, 2000). A number of studies have suggested that HCV can replicate not only in hepatocytes, but also in lymphocytes (Ducoulombier *et al.*, 2004; Karavattathayyil *et al.*, 2000, Lerat *et al.*, 1998), whereas the determinants of HCV tropism are still unknown. The development of HCV strain JFH-1, which generates infectious HCV in culture, has made an important contribution to the study of the HCV life cycle (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005). The HCV life cycle is divided into several steps. After entry into the cell and uncoating, the HCV life cycle leads to translation, polyprotein processing, RNA replication, virion assembly, transport and release. The JFH-1 subgenomic replicon can replicate in non-hepatic cell lines, such as HeLa cells and 293 cells, suggesting that the host factors required for HCV replication are not hepatocyte-specific (Kato *et al.*, 2005b). The SB strain of HCV (genotype 2b strain) was isolated from an HCV-infected non-Hodgkin's B-cell lymphoma and has been reported to infect B and T cells (Kondo *et al.*, 2007; Sung *et al.*, 2003). The virus titres of the SB strain in lymphocytes were, however, lower than those of JFH-1 in Huh-7 cells and the expression of HCV proteins was not confirmed (Kondo *et al.*, 2007). It is unknown whether HCV JFH-1 can infect and replicate in lymphocytes. To gain more insight into the tissue tropism of HCV infection, we investigated the infection, replication, IRES-dependent translation and polyprotein processing of the JFH-1 strain in nine lymphocytic cell lines.

We first sought to determine whether HCV JFH-1 can infect lymphocytic cell lines. We chose nine lymphocytic cell lines derived from Burkitt's lymphoma, the EBV-immortalized human B cell line, lymphoblasts and acute T-cell leukaemia. C1R, IB4, Namalwa, P3HR1 and Raji cells were Epstein–Barr virus (EBV)-positive (Table 1). Infectious HCV was generated from HCV JFH-1 RNA in Huh-7 cells (Shirakura *et al.*, 2007; Wakita *et al.*, 2005) and the calculation of the 50 % tissue culture infectious dose ($TCID_{50}$) was based on methods described previously (Lindenbach *et al.*, 2005). These cell lines (1×10^5 cells per well of a six-well plate) were incubated with 2 ml inoculum (5×10^3 or 5×10^4 $TCID_{50}$ ml^{-1}) for 3 h, washed three times with PBS, and cultured in fresh medium. The culture medium was changed every 2 days. Cells were harvested at 0 (3 h post-infection [p.i.]), 4 and 8 day p.i. HCV core antigen within cells was quantified by immunoassay (Ortho HCV-core ELISA kit; Ortho-Clinical Diagnostics). As shown in Fig. 1(a), increasing the HCV titre of the inoculum resulted in a 7.2-fold increase in the levels of HCV core protein in Huh-7 cells at 3 h p.i. Increasing the HCV titre of the inoculum resulted in a 1.5- to 3.2-fold increase in the levels of the core protein in C1R, BL41,

Table 1. Summary of the virological characterization of HCV JFH-1 in lymphocytes

Name	Source	EBV	Transfection			Concentration of G418 for selection (μg ml^{-1})	HCVcc infection	HCV-RNA replication	Translation*		Polyprotein processing†
			Buffer	Program	Efficiency				HCV-IRES	EMCV-IRES	
Bjab	Burkitt's lymphoma	−	T	T−16	>90 %	600–800	−	−	+	+ +	+
BL41	Burkitt's lymphoma	−	V	I−10	60–70 %	1000	−	−	+	+ +	ND
C1R	B lymphoblast	+	V	T−20	70–80 %	100	−	−	+ + + +	+ + + +	+
IB4	Lymphoblastoid	+	V	T−20	80–90 %	1000	−	−	+ + +	+ + + +	+
Jurkat	Acute T cell leukaemia	−	V	I−10	60–70 %	600	−	−	+ +	+	ND
Namalwa	Burkitt's lymphoma	+	V	M−13	60–70 %	600–800	−	−	+ + +	+ + + +	+
P3HR1	Burkitt's lymphoma	+	V	A−23	60–70 %	800	−	−	+ + +	+ + + +	ND
Raji	Burkitt's lymphoma	+	V	T−27	70–80 %	800	−	−	+ +	+ + + +	+
Ramos	Burkitt's lymphoma	−	V	M−13	40–60 %	400	−	−	+	+	ND
Huh7	Hepatoma	−	T	T−14	70–80 %	500	+	+	+ + +	+ + +	+

*+, <0.25 fold IRES activity of Huh-7; + +, 0.25–0.75 fold; + + +, 0.75–1.5-fold; + + + +, >1.5-fold.
†ND, Not determined.

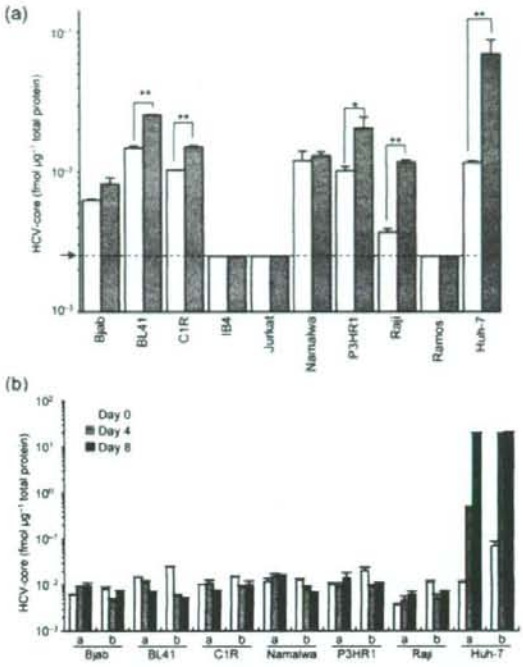

Fig. 1. HCV infection assay. (a) HCV core protein levels 3 h after infection. A total of 1×10^5 cells were infected with 2 ml of the inoculum (5×10^3 [white bars] or 5×10^4 [grey bars] $TCID_{50}$ ml^{-1}) for 3 h at 37 °C and harvested at 3 h p.i. HCV core protein in cell lysate was quantified by ELISA. The average values with standard deviations from triplicate samples are shown. The cut-off value of the immunoassay is indicated by an arrow and a dotted line. The difference between low m.o.i. (white bars) and high m.o.i. (grey bars) was significant (*, $P<0.05$; **, $P<0.01$, Student's *t*-test). (b) Time-course of HCV core protein levels after infection. In total, 1×10^5 cells were infected with 2 ml of the inoculum (5×10^3 [a] or 5×10^4 [b] $TCID_{50}$ ml^{-1}) for 3 h and harvested at 0, 4 and 8 days p.i. HCV core protein in cell lysate was quantified by ELISA. Average values ± SD from triplicate samples are shown.

P3HR1 and Raji cells, suggesting that HCV can bind to these cell lines (Fig. 1a). In contrast, the levels of HCV core protein in IB4, Jurkat and Ramos cells at 3 h p.i. were below the detection limits and there were no significant differences in the levels of the core protein in Bjab cells and Namalwa cells, suggesting that HCV binding to these cells was very inefficient (Fig. 1a). Moreover, the levels of HCV core protein increased in Huh-7 cells but, in the case of all lymphocytic cell lines, including Raji cells, the core titre did not increase at day 4 and 8 p.i., suggesting that HCV JFH-1 does not infect and/or replicate efficiently in these lymphocytic cell lines (Fig. 1b).

To assess the replication of JFH-1 in our lymphocytic cell lines, we utilized the HCV replicon system. To visualize the

replicating cells, a reporter replicon plasmid was constructed as follows. The gene encoding green fluorescence protein (GFP) was fused to the neomycin resistance gene using an overlap PCR amplification technique and the fusion product was inserted into pSGR-JFH1. The resultant plasmid was pSGR-GFPneo-JFH1. This plasmid was linearized with *Xba*I and used as a template for *in vitro* transcription using an AmpliScribe T7 High Yield Transcription kit (Epicentre Biotechnologies). RNA was transfected with high transfection efficiency and low cytotoxicity using the Nucleofector system (Amaxa Biosystems) (Coughlin *et al.*, 2004; Miyahara *et al.*, 2005; Van De Parre *et al.*, 2005). The transfection efficiencies ranged from 60 to 80 % after optimization of transfection conditions (Table 1). GFP expression was monitored periodically during the selection of HCV-replicon cells by G418 (Table 1). The GFP-expressing cells were detected at day 3 post-transfection (p.t.) in Huh-7, P3HR1, Raji, C1R and Namalwa cells. The rate of GFP expression in Huh-7 cells was more than 50 %. The rate of GFP-expression in lymphocytic cell lines was less than 1 %, despite the high transfection efficiencies. After 3 weeks of G418 selection, SGR-GFPneo-JFH1 replicon cells were established in Huh-7 cells, but not in lymphocytic cells. These data suggest that JFH-1 subgenomic replicon RNA cannot replicate in the lymphocytic cell lines.

To facilitate quantification of replication, we performed luciferase assays using subgenomic replicon RNA (SGR-JFH1/Luc) carrying firefly luciferase as a reporter. SGR-JFH1/Luc RNA was *in vitro*-transcribed using the linearized pSGR-JFH1/Luc (Kato *et al.*, 2005a) as template DNA. Cells were harvested at 4, 24, 48 and 72 h p.t. and luciferase activities were assayed with luciferase assay reagent (Promega). Assays were performed at least in triplicate. There were significant differences in luciferase activities at 4 h p.t. among the cell lines, probably because there were differences in transfection efficiencies and the doubling time of the cell lines. Thus, the replication activity was expressed relative to the reporter activity determined 4 h p.t. for each cell line, which was set to 1 (Fig. 2a). HCV subgenomic replicon RNA efficiently replicated in Huh-7 cells (Fig. 2a). Replication-deficient subgenomic replicon RNA encoding a GDD to GND mutation in NS5B served as a negative control in Huh-7 cells. The luciferase activities of replication-deficient subgenomic replicon RNA in lymphocytic cell lines also decreased rapidly (data not shown). As shown in Fig. 2(a), the luciferase activities of HCV subgenomic replicon RNA in lymphocytic cell lines decreased rapidly, suggesting that HCV subgenomic replicon RNA did not replicate efficiently in lymphocytic cell lines. Thus, these two different replicon assays demonstrated that the HCV JFH-1 subgenomic replicon failed to replicate in our lymphocytic cell lines.

To determine which steps of the HCV life cycle are impaired, we further examined translation and polyprotein processing. At first, we assessed HCV IRES-dependent translational efficiencies in the lymphocytic cell lines. Cells were co-transfected with the subgenomic replicon RNA (SGR-JFH1/Luc) and a capped RNA encoding *Renilla* luciferase (cap-luc). Cap-luc RNA was *in vitro*-transcribed using a T7 mMessage mMachine kit (Ambion). The HCV IRES activities in IB4, Namalwa and P3HR1 cells were as high as in Huh-7 cells. The HCV IRES activities in Jurkat and Raji cells were about 50 % of those in Huh-7 cells, and the HCV IRES activities in Bjab, BL41 and Ramos cells were less than 25 % of those in Huh-7 cells. On the other hand, the HCV IRES activity in C1R cells was about twofold higher than in Huh-7 cells (Fig. 2b). Replication-deficient subgenomic replicon RNA encoding a GDD to GND mutation in NS5B showed a luciferase activity level similar to that of the wild-type, suggesting that the luciferase activity at 4 h after transfection reflected translational levels but not replication levels (data not shown). Our data indicate high HCV IRES activities in all cell lines, except in Bjab, BL41 and Ramos.

The HCV polyprotein is translated in subgenomic replicon cells in an encephalomyocarditis virus (EMCV) IRES-dependent manner. To rule out the possibility that the EMCV IRES-dependent translation is impaired in lymphocytic cell lines, we assessed the EMCV IRES-dependent translational efficiencies. We assayed EMCV IRES activity using EMCV IRES-driven luciferase RNA (EMC-luc) and Cap-luc RNA. The EMCV IRES activity was five- to tenfold higher in C1R, Namalwa, IB4 and P3HR1 than in Huh-7 cells (Fig. 2c). From these results, HCV IRES and EMCV IRES exhibited sufficient translational activity in C1R, Namalwa, P3HR1 and Raji cells, suggesting that IRES-dependent translation was not impaired in these lymphocytic cell lines.

To determine whether HCV polyprotein is properly processed in lymphocytes, we examined the processing of HCV non-structural (NS) proteins. The construct pSGR-JFH1/Luc expresses the polyprotein NS3-NS4A-NS4B-NS5A-NS5B. The HCV NS3/4A protease is responsible for proteolytic processing at each cleavage site. We used the eukaryotic transient-expression system based on a recombinant vaccinia virus carrying bacteriophage T7 RNA polymerase (T7vac) (Fuerst *et al.*, 1989). To express the SGR-JFH1/Luc encoding HCV NS proteins, 5×10^6 cells were transfected with 5 μg pSGR-JFH1/Luc and infected with 2.5×10^9 p.f.u. T7vac, harvested at 24 h p.i., and analysed by Western blotting. Completely processed NS3, NS5A and NS5B proteins were detected in Bjab, Raji, IB4 and Namalwa cells as well as in pSGR-JFH1/Luc-transfected Huh-7 cells and HCV-JFH1-infected Huh-7 cells (Fig. 2c). The unprocessed polyprotein was not detected by immunoblotting in these lymphocytic cell lines (data not shown). These results suggest that the HCV polyprotein is efficiently processed in these lymphocytic cells.

In this study, we demonstrated that HCV JFH-1 failed to infect and replicate in nine lymphocytic cell lines. In contrast, HCV IRES-dependent translation and polyprotein processing by NS3/NS4A protease functioned properly

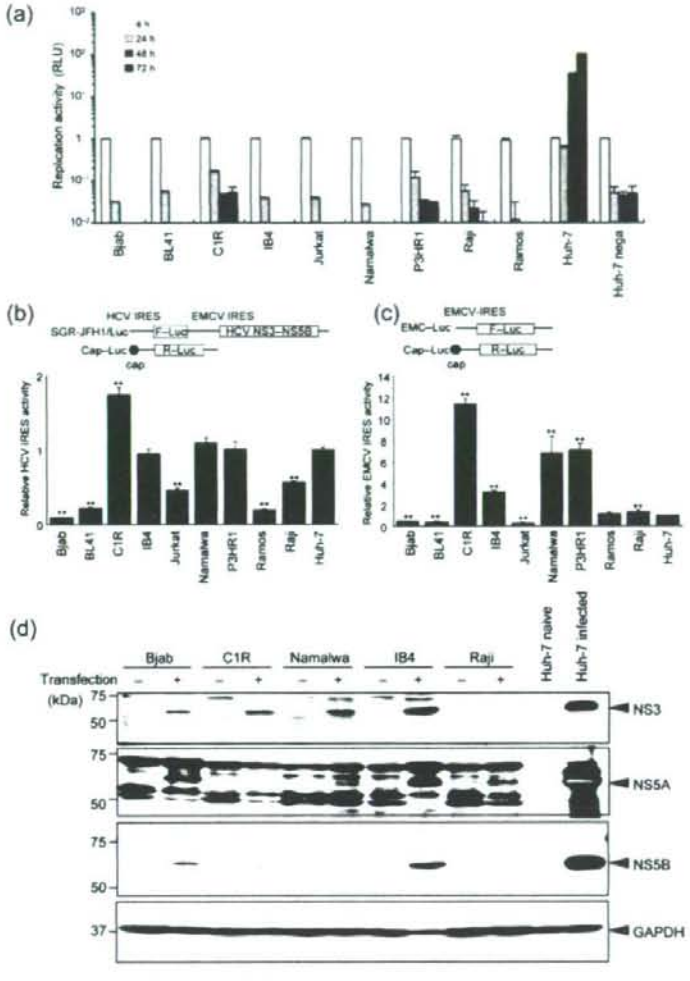

Fig. 2. Replication, HCV IRES-dependent translational efficiencies and polyprotein processing. (a) Subgenomic replicon assay. JFH-1 subgenomic replicon RNA was transfected into several cell lines and harvested at 4, 24, 48 and 72 h p.t. The replication activity was expressed relative to the reporter activity determined 4 h p.t. for each cell line, which was set to 1. RLU, Relative luciferase units; Huh-7 nega, Huh-7 cells transfected with SGR-JFH1/Luc GND, served as a negative control. (b) HCV IRES-dependent translational efficiency. To determine the HCV IRES activities, we co-transfected cells with SGR-JFH1/Luc RNA and Cap-Renilla luciferase RNA. The IRES activity of each cell line is expressed in relation to Huh-7 IRES activity, that is, as the ratio of HCV IRES-driven firefly luciferase activity to cap-driven *Renilla* luciferase activity. The difference in HCV IRES activity between Huh-7 cells and the lymphocytic cell line was significant (**, $P<0.01$, Student's *t*-test). (c) EMCV IRES-dependent translational efficiency. To determine the EMCV IRES activities, we co-transfected cells with EMCV–firefly luciferase RNA and Cap-Renilla luciferase RNA. The IRES activity of each cell line is expressed in relation to Huh-7 IRES activity, that is, as the ratio of EMCV IRES-driven firefly luciferase activity to cap-driven *Renilla* luciferase activity. The difference in EMCV IRES activity between Huh-7 cell and the lymphocytic cell line was significant (**, $P<0.01$, Student's *t*-test). (d) Polyprotein processing by NS3/4A protease in lymphocytic cell lines. pSGR-JFH1/Luc-transfected cells were infected with T7vac and harvested at 24 h p.i. HCV NS proteins, NS3, NS5A and NS5B were detected by using anti-NS3 rabbit polyclonal antibody (PAb), anti-NS5A rabbit PAb and anti-NS5B rabbit PAb. Arrowheads indicate the processed NS3, NS5A and NS5B proteins, respectively.

in these cells. Moreover, subgenomic replicon RNA failed to replicate in these cell lines. Our data suggest that lymphocytic cell lines may lack some host factors required for infection and replication of HCV-JFH1.

Viral entry often requires sequential interactions between viral proteins and several cellular factors. Several molecules (CD81, Claudin-1, Scavenger receptor class B member IR, LDL-receptor and glycosaminoglycans) have been reported to be involved in HCV binding and entry (Barth *et al.*, 2003; Evans *et al.*, 2007; Pileri *et al.*, 1998; Scarselli *et al.*, 2002). Further investigation will be required to clarify HCV binding and entry into lymphocytic cell lines.

HCV IRES and EMCV IRES exhibited sufficient translational activities in C1R, IB4, P3HR1, Namalwa and Raji cells. All these cell lines are EBV-positive. EBV-encoded nuclear antigen (EBNA1) has been reported to support HCV replication (Sugawara *et al.*, 1999). Two small EBV-encoded RNA species (EBERs) bind to the HCV IRES region (Wood *et al.*, 2001). These findings raise the possibility that HCV IRES activities may be modified by the EBV genome.

HCV JFH-1 subgenomic replicon RNA could not replicate in all lymphocytes tested in this study. The HCV SB strain, however, has been reported to infect Raji, Daudi, Molt-4

and Jurkat cells (Kondo *et al.*, 2007; Sung *et al.*, 2003). Still unknown is how hepatotropism and lymphotropism of HCV are determined. The GB virus B (GBV-B) is most closely related to HCV and the GBV-B infection of tamarins has been proposed as a good surrogate model for chronic hepatitis C (Bukh *et al.*, 2001; Jacob *et al.*, 2004; Lanford *et al.*, 2003; Martin *et al.*, 2003). A recent report has shown that GBV can disseminate to not only liver but also a variety of extrahepatic tissues such as haematolymphoid and genital tissues in tamarins (Ishii *et al.*, 2007). Viral RNA cloned from plasma and liver from the tamarins showed no sequence heterogeneity, suggesting that host factors determine the pleiotropism (Ishii *et al.*, 2007). It remains unclear how host factors and/or viral factors determine the tissue tropism of HCV. Further studies will be required to clarify the molecular mechanisms of HCV tissue tropism.

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Original Article

Effect of treatment with interferon α-2b and ribavirin in patients infected with genotype 2 hepatitis C virus

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Aim: Nearly 20% of chronic hepatitis C (CHC) patients with genotype 2 hepatitis C virus (HCV) infection are not curable, even by interferon (IFN)–ribavirin combination therapy. The aim of this study is to investigate the factors that determine the efficacy of combination therapy in patients with genotype 2 HCV infection.

Methods: Fifty patients with CHC who underwent a treatment of 6 MU IFN α-2b with ribavirin for 24 weeks were retrospectively analyzed.

Results: All the patients showed no serum HCV-RNA within 12 weeks after starting the therapy. Forty-one of the 50 patients (82%) achieved a sustained virological response (SVR). The age, sex, genotype (2a vs. 2b) and grade/stage of the liver by histopathology and pretreatment viral load were not different between the sustained responders and relapsers. Univariate analysis showed that an earlier viral clearance from blood and a larger number of amino acid substitutions in the interferon sensitivity determining region (ISDR) were predictors of SVR. Multivariate analysis showed that a large number of amino acid substitutions in the ISDR was a predictor of SVR.

Conclusion: The characterization of the amino acid sequences of ISDR may be helpful for predicting a relapse after combination therapy in patients with genotype 2 HCV infection.

Key words: genotype, hepatitis C virus, interferon, ISDR, ribavirin

INTRODUCTION

CHRONIC HEPATITIS C (CHC) is an infection that affects more than 150 million people worldwide. Up to 50% of these people develop chronic liver disease leading to liver cirrhosis.[1–3] Once liver cirrhosis has developed, up to 7% of these patients per year develop hepatocellular carcinoma.[4–6] Antiviral treatment is crucial for the control of this disease.

Before the use of ribavirin, interferon (IFN) monotherapy was the only effective treatment for CHC. Although many clinical trials and several meta-analyses have documented the efficacy of IFN monotherapy, the rate of sustained virological response (SVR) is low, particularly in patients with genotype 1 or 4 hepatitis C virus (HCV) infection.[7–9]

The combination therapy of IFN and ribavirin has been shown to be more effective than IFN monotherapy for CHC.[10–14] The baseline level of serum HCV-RNA before treatment and HCV genotype are predictors of a SVR to IFN therapy.[15] With regard to HCV genotype, patients who are infected with genotype 2 or 3 HCV can achieve a higher SVR rate than those with genotype 1 HCV. However, even with genotype 2 HCV infection, combination therapy for 24 weeks failed to eradicate the virus in about 20% of patients,[12–14] although the reason for this is still unclear.

Besides HCV genotype and viral load, mutations in the interferon sensitivity determining region (ISDR, aa 2209–2248) of the non-structural region 5A (NS5A) of

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HCV have also been reported to influence the efficacy of IFN. In genotype 1 HCV infection, the number of amino acid substitutions in ISDR is reported to be related to the efficacy of IFN therapy in Japan and Europe,[16–20] although this correlation is still controversial.[21] In genotype 2 infection, the amino acid sequence of ISDR has been reported to also correlate with SVR to IFN monotherapy.[22–24] Therefore, the efficacy of IFN–ribavirin combination therapy in genotype 2 HCV-infected patients may be determined by the amino acid sequence of ISDR, which has not yet been studied.

The aim of this study is to elucidate factors that determine the response to IFN–ribavirin combination therapy in patients with genotype 2 HCV infection.

METHODS

Patient selection

FROM 2001 TO 2003, 140 patients (84 men and 56 women; mean age, 53.8 ± 11.3 years) were treated with recombinant IFN α-2b (Intron A; Schering-Plough, Kenilworth, NJ) and ribavirin (Rebetol; Schering-Plough, Kenilworth, NJ) combination therapy. Eighty-five patients had genotype 1 HCV infection (54 men and 31 women; mean age, 56.3 ± 10.5 years) and 55 patients had genotype 2 HCV infection (30 men and 25 women; mean age, 50.0 ± 11.6 years). All the patients with genotype 2 HCV infection were treated daily with IFN α-2b at 6 MU for two weeks, followed by treatment three times a week with IFN α-2b 6 MU for 22 weeks in combination with ribavirin. Ribavirin was given orally twice a day at a total daily dose of 600 mg for 24 weeks for patients who weighed 60 kg or less and 800 mg for patients who weighed more than 60 kg. Fifty of the 55 patients with genotype 2 HCV infection with available clinical data were retrospectively analyzed.

HCV markers

HCV genotype was determined by a direct sequencing of the amplified products generated during the Amplicor Monitor test (Roche Diagnostics, Branchburg, NJ)[25] with an ABI 3700 DNA sequencer (Perkin Elmer, Applied Biosystems, Foster City, CA).[26] HCV-RNA level was determined using Amplicor-M version 2 (Chugai-Roche Diagnostics, Tokyo, Japan).

Polymerase chain reaction (PCR) and determination of sequences of ISDR

Complementary DNA (cDNA) was prepared by reverse transcription using an RNA–PCR kit (Takara Bio, Shiga, Japan). In brief, 1 μL of RNA solution, extracted from 100 μL of serum and dissolved in 25 μL of RNase-free distilled water, was mixed with 4 μL of 1.5 mM $MgCl_2$ solution, 2 μL of 10× RNA–PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl), 8.5 μL of RNase-free distilled H_2O, 2 μL of a dNTP mixture (10 mM dATP, dCTP, dGTP, dTTP), 1 μL of random 9-mers (5′-NNNNNNNNN-3′), 0.5 μL of RNase inhibitor (Takara Bio, Shiga, Japan) and 1 μL of reverse transcriptase (Takara Bio, Shiga, Japan), was reverse transcribed at 42°C for 30 min.

The first round PCR was performed using the external primers (sense primer; nt 6824–6846; 5′-TCTCAG CTCCCTTGCGATCCTGA-3′ and antisense primer; nt 7155–7139; 5′-GATGGTATCGAAGGCTC-3′) and 2.5 U of Ex Taq polymerase (Takara Bio, Shiga, Japan) with proofreading activity. The amplification conditions consisted of 94°C for 16 min followed by 40 cycles of 94°C for 1 min, 50°C for one minute and 72°C for one minute. One microliter of the first PCR product was used for the second PCR with internal primers (sense primer; nt 6950–6968; 5′-AGCTCCTCA GCGAGC CAGCT-3′, and antisense primer; nt 7104–7085; 5′-GATGGTATCGAAGGCTC-3′) and 0.5 μL of amplitaq gold (Roche Diagnostics, Branchburg, NJ). The amplification conditions of the second PCR were the same as those of the first PCR. The second PCR products were analyzed by 2% agarose gel electrophoresis, stained with ethidium bromide and visualized by UV transillumination.

Amplification products were purified on Wizard PCR Preps DNA purification resin (Promega, Madison, WI) and sequenced bidirectionally with the Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer, Applied Biosystems, Foster City, CA) using the above PCR primers. Sequencing was performed using an automated DNA sequencer ABI 377 (Perkin Elmer, Applied Biosystems, Foster City, CA).

Histopathology

A liver biopsy was performed on each patient within six months before the start of therapy. The histopathological findings were assessed by grading inflammatory activity and the staging of fibrosis using the classification of Desmet *et al.*[27] by an experienced pathologist who had no knowledge of the clinical data of the patients.

Statistical analysis

The collected data were analyzed using the SPSS program, version 11.0J (SPSS, Chicago, IL). The distributions of continuous variables were analyzed using the

Table 1 Clinical background of patients

	Genotype of HCV			Difference
	2 ($n = 50$)	2a ($n = 32$)	2b ($n = 18$)	P (2a vs. 2b)
Age (years)	49.2 ± 11.8	50.6 ± 10.1	46.6 ± 12.2	0.25
Male	30 (60%)	20 (63%)	10 (56%)	0.63
Viral load (KIU/mL)	491.6 ± 286.2	420.3 ± 264.8	618.2 ± 279.0	0.02
Histopathology				
Grade (0/1/2/3)	0/29/17/2	0/16/13/1	0/13/4/1	0.34
Stage (0/1/2/3/4)	1/23/14/9/1	1/10/10/8/1	0/13/4/1/0	0.02
SVR	41 (82%)	27 (84%)	14 (78%)	0.15

SVR, sustained virological response.

Mann–Whitney *U*-test. Differences in proportions were tested using Fisher's exact test. Independent factors that may influence the response to combination therapy were identified using stepwise multiple logistic regression analysis. Variables with $P < 0.1$ at univariate analysis were retained for the multivariate logistic regression analysis. The significance of correlation was evaluated by Spearman's rank analysis. A two-tailed *P*-value of <0.05 was considered to indicate statistical significance.

RESULTS

Baseline characteristics of treated patients

TABLE 1 SHOWS the clinical background of the treated patients with genotype 2 HCV infection. The patients comprised 30 men and 20 women with a mean age of 49.2 ± 11.8 years. The patients with genotype 2a have lower viral loads and more severe fibrosis than those with genotype 2b HCV infection. The rate of SVR was 84% (27 of 32) in the patients with genotype 2a and 78% (14 of 18) in those with genotype 2b.

Amino acid sequence of ISDR

The amino acid sequence of ISDR was determined in 29 of the 32 patients with genotype 2a and 17 of the 18 patients with genotype 2b. The number of amino acid substitutions in ISDR was positively correlated with viral load (Spearman's rank correlation coefficient $r = -0.53$, $P < 0.001$). Figure 1 shows the amino acid sequences of ISDR. The prototype sequences of genotype 2a (D10749)[28] and 2b (D10988)[29] were determined to be the reference sequence for genotype 2a and 2b, respectively. The rate of SVR in the patients with no amino acid substitutions (wild type) in their ISDR sequence was 57% (8/14). In the patients with one to three amino acid substitutions (intermediate) and four or more substitutions (mutant) in their ISDR sequences, the rates of SVR were 85% (22/26) and 100% (8/8), respectively. In the patients with genotype 2a HCV infection, the rates of SVR in the wild, intermediate and mutant type ISDR were 63% (5/8), 80% (12/15) and 100% (8/8), respectively. In genotype 2b HCV infection, the rate of SVR in wild and intermediate type ISDR was 50% (3/6) and 91% (10/11), respectively.

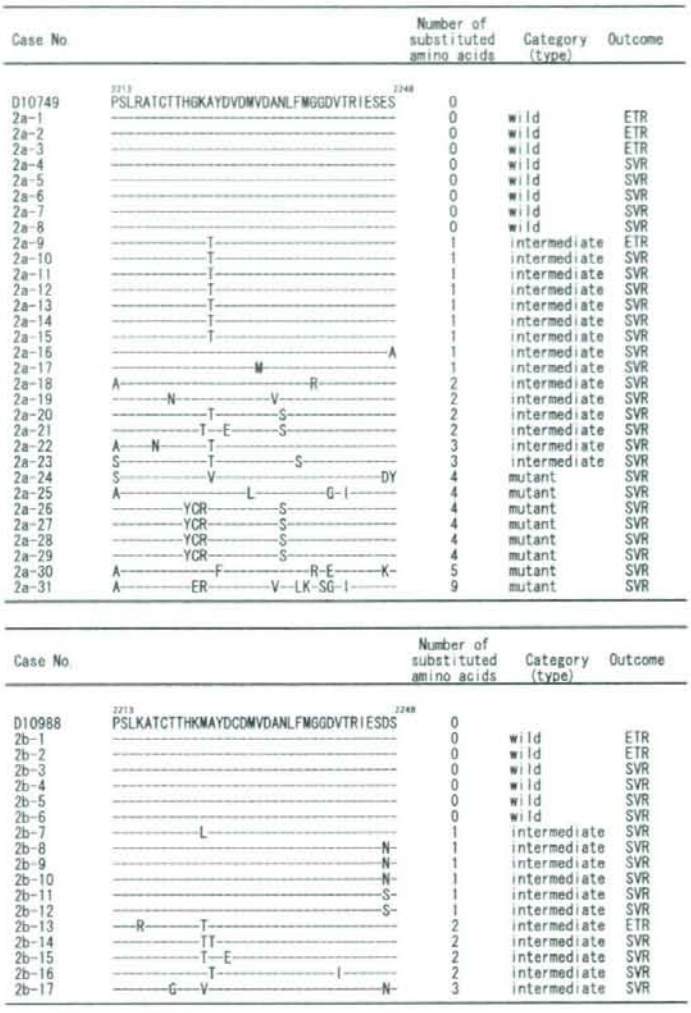
Predictors of response

The characteristics of patients with SVR and those without were compared (Table 2). By univariate analysis, time of viral clearance from blood ($P = 0.018$) and

Table 2 Univariate logistic regression analysis for factors responsible for sustained virological response

	SVR	non-SVR	Univariate analysis *P*	Odds ratio
Age	51 (22–68)	52 (28–63)	0.805	0.992
Gender	21:17	7:2	0.195	0.329
Genotype (2a vs. 2b)	25:13	5:4	0.561	1.636
Histology of liver				
Grading (0/1/2/3)	0/21/16/2	0/8/1/0	0.086	6.438
Staging (0/1/2/3/4)	1/18/12/7/1	0/5/2/2/0	0.897	1.058
Pretreatment viral load (KIU/mL)	430 (8.7–>850)	710 (480–>850)	0.323	0.999
Time of viral clearance from blood (days)	14 (7–70)	52 (28–63)	0.018	0.649
Number of substituted amino acids in ISDR	1 (0–1)	0 (0–2)	0.048	3.716

SVR, sustained virological response.



Case No.	2213 PSLRATCTTHGKAYDVDMVDANLFMGGDVTRIESES 2248	Number of substituted amino acids	Category (type)	Outcome
D10749		0		
2a-1		0	wild	ETR
2a-2		0	wild	ETR
2a-3		0	wild	ETR
2a-4		0	wild	SVR
2a-5		0	wild	SVR
2a-6		0	wild	SVR
2a-7		0	wild	SVR
2a-8		0	wild	SVR
2a-9	----------T----------	1	intermediate	ETR
2a-10	----------T----------	1	intermediate	SVR
2a-11	----------T----------	1	intermediate	SVR
2a-12	----------T----------	1	intermediate	SVR
2a-13	----------T----------	1	intermediate	SVR
2a-14	----------T----------	1	intermediate	SVR
2a-15	----------T----------	1	intermediate	SVR
2a-16	----------A	1	intermediate	SVR
2a-17	----------M----------	1	intermediate	SVR
2a-18	A----------R----------	2	intermediate	SVR
2a-19	----------N----------V----------	2	intermediate	SVR
2a-20	----------T----------S----------	2	intermediate	SVR
2a-21	----------T--E-----S----------	2	intermediate	SVR
2a-22	A---N-----T----------	3	intermediate	SVR
2a-23	S----------T----------S----------	3	intermediate	SVR
2a-24	S----------V----------DY	4	mutant	SVR
2a-25	A----------L----------G-I----------	4	mutant	SVR
2a-26	----------YCR----------S----------	4	mutant	SVR
2a-27	----------YCR----------S----------	4	mutant	SVR
2a-28	----------YCR----------S----------	4	mutant	SVR
2a-29	----------YCR----------S----------	4	mutant	SVR
2a-30	A----------F----------R-E-----K-	5	mutant	SVR
2a-31	A----------ER----------V--LK-SG-I----------	9	mutant	SVR

Case No.	2213 PSLKATCTTHKMAYDCDMVDANLFMGGDVTRIESDS 2248	Number of substituted amino acids	Category (type)	Outcome
D10988		0		
2b-1		0	wild	ETR
2b-2		0	wild	ETR
2b-3		0	wild	SVR
2b-4		0	wild	SVR
2b-5		0	wild	SVR
2b-6		0	wild	SVR
2b-7	----------L----------	1	intermediate	SVR
2b-8	----------N-	1	intermediate	SVR
2b-9	----------N-	1	intermediate	SVR
2b-10	----------N-	1	intermediate	SVR
2b-11	----------S-	1	intermediate	SVR
2b-12	----------S-	1	intermediate	SVR
2b-13	---R----------T----------	2	intermediate	ETR
2b-14	----------TT----------	2	intermediate	SVR
2b-15	----------T--E----------	2	intermediate	SVR
2b-16	----------T----------I----------	2	intermediate	SVR
2b-17	----------G---V----------N-	3	intermediate	SVR

Figure 1 Figures 1a and 1b show patients with genotypes 2a and 2b, respectively. The rate of sustained virological response (SVR) in patients with no amino acid substitutions in interferon sensitivity determining region (ISDR) sequence (wild type) was 57% (8/14). In patients with one to three amino acid substitutions (intermediate) and four or more substitutions (mutant) in the ISDR sequences, the rates of SVR were 85% (22/26) and 100% (8/8), respectively. ETR, end of treatment for virological response.

amino acid mutations in the ISDR ($P = 0.048$) were found to be significantly linked to SVR. Because these variables were mutually correlated, multivariate analysis including histological grading was performed. In the final step, amino acid mutations in the ISDR (odds ratio [OR], 4.280; 95% confidence interval [CI], 1.139–16.038; $P = 0.031$) entered the model and could not be removed (Table 3). Therefore, amino acid mutations in ISDR are the only factor associated with SVR.

DISCUSSION

IN JAPAN, THE combination therapy of IFN and ribavirin for 24 weeks was approved in late 2001. It was shown that approximately 20% of patients infected with genotype 1b HCV with a high viral load attained SVR with this regimen.[30] Compared to those with genotype 1, patients with genotype 2 or 3 HCV infection are expected to achieve higher SVR rates.[12–14] However,

Table 3 Multivariate logistic regression analysis for factors responsible for sustained virological response

	SVR	non-SVR	Multivariate analysis *P*	Odds ratio
Grading (0/1/2/3)	0/21/16/2	0/8/1/0	0.547	2.141 (0180–25.463)
Time of viral clearance from blood (days)	14 (7–70)	52 (28–63)	0.091	0.552 (0.277–1.100)
Number of substituted amino acids in ISDR	1 (0–1)	0 (0–2)	0.031	4.280 (1.139–16.038)

ISDR, interferon sensitivity determining region; SVR, sustained virological response.

information on individual genotypes, in particular genotype 2, is quite limited,[31] which prompted us to conduct this study.

In this study the SVR rate of patients with genotype 2 was 82%, which is lower than that found in a previous report by Zeuzem *et al.*[31] According to the data of previous studies,[32,33] a high SVR rate may be expected in genotype 2 or 3 even if the treatment period is 24 weeks. One possible reason for the low SVR rate in this study is the use of conventional IFN-α. Pegylated IFN-α is superior to conventional IFN-α for inducing sustained viral clearance.[33,34] Another possible reason is ethnicity, because response to IFN–ribavirin combination therapy varies among races.[35,36]

The number of mutations in the ISDR of NS5A is variable and influences the efficacy of IFN–ribavirin combination therapy. Studies from Japan and Europe showed that the number of amino acid substitutions in ISDR influences the efficacy of IFN monotherapy in genotype 1 infection.[16–20] The efficacy of IFN–ribavirin combination therapy in genotype 1 infection is also influenced by the amino acid sequence of ISDR.[37] In genotype 2 infection, the amino acid sequence of ISDR has been reported to also correlate with the SVR to IFN monotherapy.[22–24] Our results suggest that the amino acid sequence of ISDR may also influence the efficacy of combination therapy in genotype 2 infection.

It is interesting that mutations in ISDR confer susceptibility to IFN–ribavirin combination therapy. It was reported that NS5A suppresses PKR protein kinase, a mediator of IFN-induced antiviral resistance[38] in genotype 1 infection. Multiple ISDR mutations probably abrogate this action of NS5A to inhibit PKR.[39] However, whether the mechanisms are also applicable to genotype 2 infection is still unclear and needs clarification.

Our study showed that about 20% of the patients with genotype 2 HCV infection were not cured by the combination therapy for 24 weeks. However, all of the uncured patients were relapsers, whose viral loads were cleared from the serum at the end of treatment. Therefore, it can be expected that these patients may be cured by a longer treatment, which should be studied further.

Figure 1a showed that cases 26, 27, 28 and 29, with no common infectious source, had the same mutations. Most of previous reported cases with mutant-type strains of ISDR had different amino acid sequences, which seems contradictory to our results.[22–24] However, one study showed that two of the four cases shared one mutant type sequence of ISDR.[23] These results imply that some viral strains with mutant type ISDR sequence are likely to be selected, which await further study.

To conclude, IFN–ribavirin combination therapy for 24 weeks cured 80% of the patients with genotype 2 HCV. Amino acid mutations in ISDR may determine the final outcome of the combination therapy.

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Chapter 23

Isolation of JFH-1 Strain and Development of an HCV Infection System

Takaji Wakita

Abstract

Detailed analysis of hepatitis C virus (HCV) has been hampered by the lack of an appropriate viral culture system and small animal models of infection. My group and others have recently reported the production of infectious virus after full-length HCV RNA transfection into Huh-7 cells. This system depends primarily on isolation of a JFH-1 strain from a patient with fulminant hepatitis. The JFH-1 strain belongs to genotype 2a and has high colony-formation efficiency when tested with a subgenomic replicon system. Here, I describe various protocols for isolation of the JFH-1 strain and construction of the HCV infection system. The HCV infection system contributes to our understanding of HCV virology and may permit development of novel antiviral strategies.

Key words: Fulminant hepatitis, JFH-1, patient sera, hepatocytes, nested RT-PCR, virus particles.

1. Introduction

To date, propagation of HCV in cultured cells has been difficult *(1)* for a number of reason, including low replication capacity of the virus and its tropism for highly differentiated hepatocytes. Inoculation of patient sera or plasma into cultured cells results in only a limited level of HCV replication, as determined by nested RT-PCR. This problem hindered the efforts of a number of HCV researchers, but in 1999, Lohmann et al. *(2)* were the first to report efficient replication of an HCV subgenomic replicon, in which an HCV structural region was replaced with a neomycin-resistance gene. After transfection of replicon RNA into Huh-7 hepatocellular carcinoma cells, followed by several weeks of G418 selection culture, replicons were established, and robust replicon

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RNA replication was observed in these cells. Adaptive mutations were found in most replicon genomes that increased virus replication at different levels, and some combinations of these adaptive mutations were observed to increase replication strongly *(3–5)*. Genomic replicons containing a structural region with adaptive mutations in a nonstructural region demonstrated efficient replication in transfected Huh-7 cells *(6–8)*, but viral particles were not produced from these genomic replicons. Furthermore, a full-length viral RNA genome with adaptive mutations synthesized in vitro was not infectious in chimpanzees, unlike the wild-type genome *(9)*. These results suggest that adaptive mutations enhance the replication capacity of the HCV RNA genome in cultured cells at the expense of efficient viral particle formation in cultured cells and in vivo.

The JFH-1 strain was isolated from a 32-year-old male patient *(10)*. He was admitted with acute liver failure and had serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations of 9160 IU/L and 6970 IU/L, respectively. The minimum prothrombin time was 16%. Stage II encephalopathy developed 5 days after admission, after which he was diagnosed with fulminant hepatitis. HCV RNA was detected by reverse transcription polymerase chain reaction (RT-PCR) with sera obtained during the acute phase. Anti-HCV antibody was also tested for but not detected on admission (by second-generation enzyme-linked immunosorbent assay, Ortho Diagnostics, Tokyo, Japan). All viral markers indicating exposure to other hepatitis viruses were negative. After admission, the patient's liver function and clinical condition improved with conservative treatment. Anti-HCV antibody became positive 6 weeks after admission. These findings suggest that his fulminant hepatitis was in fact due to HCV infection. The infectious strain of HCV was analyzed in 12 sets of nested RT-PCR, as well as 5′ RACE and 3′ RACE RT-PCR, which covered the entire HCV genome. All of the PCR products were cloned and sequenced. Five clones of each PCR fragment were sequenced, and the consensus sequence was determined. According to sequence analysis, the JFH-1 strain belongs to genotype 2a, and its sequence deviates slightly from other genotype 2a clones isolated from patients with chronic hepatitis (10).

Subgenomic replicon and full-length constructs were assembled with cloned PCR fragments *(11–13)*. The colony-formation efficiency of the JFH-1 replicon was much greater than that of the Con1 replicon with adaptive mutations. Furthermore, transient transfection of replicon RNA into Huh-7 cells resulted in autonomous RNA replication, as determined by northern-blot analysis *(11, 14)*. Importantly, adaptive mutations were not necessary for efficient JFH-1 replicon replication in Huh-7 cells. In addition, the JFH-1 replicon produced colonies in several