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Dynamic behavior of hepatitis C virus quasispecies in a long-term culture of the three-dimensional radial-flow bioreactor system

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Abstract

Hepatitis C virus (HCV) exists in infected individuals as quasispecies, usually consisting of a dominant viral isolate and a variable mixture of related, yet genetically distinct, variants. A prior HCV infection system was developed using human hepatocellular carcinoma cells cultured in the three-dimensional radial-flow bioreactor (RFB), in which the cells retain morphological appearance and their differentiated hepatocyte functions for an extended period of time. This report studies the selection and alteration of the viral quasispecies in the RFB system inoculated with pooled serum derived from HCV carriers. Monitoring the viral RNA and core protein in the culture supernatants, together with nucleotide sequencing of hypervariable region 1 of the HCV genome, demonstrated that (1) the virus production intermittently fluctuated in the cultures, (2) the viral genetic diversity was markedly reduced 3 days post-infection (p.i.), and (3) dominant species changed on days 19–33 p.i., suggesting that the virus populations can be selected according to susceptibility to the viral infection and replication. A therapeutic effect of interferon-α also demonstrated the inhibition of HCV expression. Thus, this HCV infection model in the RFB system should be useful for investigating the dynamic behavior of HCV quasispecies in cultured cells and evaluating anti-HCV compounds.

Keywords: Hepatitis C virus; Three-dimensional culture; Radial-flow bioreactor; Dynamics; Quasispecies

1. Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver diseases (Choo et al., 1989; Kuo et al., 1989; Saito et al., 1990) and has been estimated to infect more than 170 million people throughout the world (Poynard et al., 2003). Symptoms of persistent HCV infection extend from chronic hepatitis to cirrhosis and ultimately hepatocellular carcinoma (Choo et al., 1989; Kuo et al., 1989; Saito et al., 1990). HCV belongs to the genus *Hepacivirus*, included in the family of Flaviviridae, and possesses a viral genome of a single, positive-stranded RNA with a nucleotide (nt) length of approximately 9.6 kb (Choo et al., 1991; Grakoui et al., 1993; Hijikata et al., 1991). It has been shown that HCV, like many other RNA viruses, circulates within infected individuals as a diverse population and closely related variants are referred to as quasispecies (Martell et al., 1992). This quasispecies model of mixed virus populations may imply a significant survival advantage because the simultaneous presence of multiple variant genomes and/or high rate of generation of new variants allow rapid selection of the mutants are better suited to new environmental conditions (Pawlotsky, 2006).

Studies on HCV replication and development of selective antiviral drugs have been hampered primarily by the lack of efficient cell culture systems. Establishment of selectable dicistronic HCV RNAs that are capable of autonomous replication to high levels in human hepatoma Huh-7 cells was a

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significant breakthrough in HCV research; however, virus production has not been observed in the conventional monolayer cultures (Blight et al., 2000; Lohmann et al., 1999). Recently, it has been described that infectious HCV particles are efficiently produced from a genotype 2a isolate JFH-1 in Huh-7 cells (Blight et al., 2000; Wakita et al., 2005; Zhong et al., 2005). This JFH-1 based HCV culture system is an invaluable achievement permitting a variety of studies on the complete HCV life cycle. However, HCV infection systems with human sera or plasmas containing intact virions are still limited because of low levels of propagation in the cultures. Reverse transcription (RT)-PCR was typically used to detect the viral RNA in cell extracts; however, synthesized viral proteins were not observed in these systems (Ikeda et al., 1998; Tagawa et al., 1995).

There are reports of differentiated human hepatoma FLC4 (functional liver cell 4) cells grown in a three-dimensional (3D) radial-flow bioreactor (RFB) that can be infected by HCV-positive serum and support viral replication (Aizaki et al., 2003). Furthermore, production and release of infectious HCV has been observed in the RFB system following transfection of FLC4 cells with *in vitro* transcribed HCV genomic RNA, as well as in a 3D system using Huh-7 cells harboring genome-length dicistronic RNAs (Murakami et al., 2006). The RFB system, in which the bioreactor column consists of a cylindrical matrix with porous bead microcarriers extended vertically, was aimed initially at developing artificial liver tissues and allows liver-derived cells to maintain morphological appearance as well as their physiological functions, such as the ability to synthesize albumin and drug-metabolizing activity mediated by cytochrome P450 (Iwahori et al., 2003). The radial-flow configuration permits full contact between culture medium and cells at a physiologic perfusion flow rate, and prevents excessive shear stresses and buildup of waste products, thus ensuring the long-term viability of 3D cell culture.

The aim of the present study was to characterize HCV dynamics in the RFB system during long-term cultures inoculated with pooled serum obtained from HCV carriers, and to examine the therapeutic effects of interferon-alpha (IFN-α) in this HCV infection model.

2. Materials and methods

2.1. Cell cultures

FLC4 cells (Aoki et al., 1998), which were derived from human hepatocellular carcinoma cells and negative for HCV RNA and HBV DNA, were maintained in serum-free ASF104 medium (Ajinomoto, Japan) supplemented with 4 g/L D-glucose on the collagen-coated dishes before inoculating into the RFB column. The RFB system (ABLE, Japan) was manipulated as described previously (Aizaki et al., 2003) with minor modifications. Briefly, RFB columns, which have bed volumes of 30 or 4 mL and are filled with porous glass microcarriers (diameter 0.6 mm, vacant capacity 50%, pore size <120 μm) (Hongo et al., 2005), were seeded with FLC4 cells, which subsequently attached to the surface and inside of porous glass beads. ASF104 medium containing 2% fetal calf serum was added at a flow rate of 50 mL/day, and the culture condition was automatically controlled by monitoring temperature, pH value and oxygen levels in the vessel throughout the duration of the study.

2.2. Infection of HCV-positive sera

HCV antibody-positive sera used in this study were blood donor samples supplied by The Japanese Red Cross Center, Tokyo, Japan. HCV RNA loads in the sera were as follows: serum A, 2.4×10^6 copies/mL; serum B, 8.6×10^6 copies/mL; serum C, 5.9×10^6 copies/mL; serum D, 2.5×10^6 copies/mL; serum E, 1.0×10^7 copies/mL; serum F, 1.4×10^7 copies/mL (Table 1). In the first experiment (Fig. 3), aliquots of each serum containing 2×10^6 copies of HCV RNA were mixed and pooled serum sample with 1.2×10^7 copies was prepared as an inoculum. The pooled serum (2.5 mL) was added to the 3D cultured-FLC4 cells in the 30-mL RFB column, and the culture medium was changed after 12 h of incubation. At various times during the culture period, culture medium (50 mL) was collected to determine HCV RNA and the core protein. Collected culture media were passed through a 0.20-μm filter to remove the debris, and stored at −80 °C. In the second experiment to evaluate a therapeutic effect of anti-HCV drug (Fig. 4), 4-mL RFB columns were used. IFN-α (Sumiferon 300; Sumitomo Pharmaceuticals, Japan) was added to one of two columns at a final concentration of 100 IU/mL after the infection. Culture medium was periodically collected for determination of HCV RNA, the core protein and transaminases, and was replaced with the same volume of fresh medium with or without IFN-α.

2.3. Quantitation of HCV RNA and core protein

HCV RNA was extracted from 140 μL of each serum or culture medium using QIAamp Viral RNA Mini spin column (QIAGEN); RNA was eluted in 60 μL of water and stored at −80 °C. Real-time RT-PCR was performed using TaqMan EZ RT-PCR Core Reagents (PE Applied Biosystems), as described previously (Aizaki et al., 2003; Suzuki et al., 2005). The viral core antigen in the culture medium was quantified by immunoassay (Ortho HCV-Core ELISA Kit; Ortho-Clinical Diagnotics), according to the manufacturer's instruction (Murakami et al., 2006).

2.4. PCR amplification and nucleotide sequencing of HVR1 domain and its flanking region

Five microliters of RNA samples prepared as above were reverse transcribed using SuperScript II (Invitrogen) and a specific primer 5′-CATCCATGTGCAGCCGAACC-3′ (corresponding to nucleotides [nt] 2006–1987 of HCV NIHJ1) (Aizaki et al., 1998). For the nested PCR, a genotype-independent set of primers specific for hypervariable region 1 (HVR1). The first round of PCR was performed with the outer sense primer 5′-GCATGGCTTGGGATATGATG-3′ (nt 1291–1310) and with the reverse transcription primer described above as the outer antisense primer. After the initial 3.5-min denaturation step at 94 °C, 35 PCR cycles, with each cycle

Table 1
HCV-positive sera used in this study

Serum	Clone	HCV HVR1 sequence	% in the serum	genotype
A	A1	KVLIVMLSFAGVDGSTRTIGGRTAHTTQGSASLFSSGPAQKIQLINTNGS	75	1
	A2	-------L------N-H-V--AV-SS---FT---KL----------S---	12.5	
	A3	-------L------N-YAS---AGLL-R-V--I-TA----------S---	12.5	
B	B1	KVVVILLLAAGVDAGTNTIGGSAAQTTSGFTGLFRSGARQNIQLINTNGS	50	2
	B2	----------R	12.5	
	B3	----------S---------	12.5	
	B4	--L-V---F-----E-HVT--N-GR--A-LV--LTP--K----------	12.5	
	B5	--I----------	12.5	
C	C1	KVLIVMLLFAGVDGDTHVSGGTQGRAAYGLASLFALGPTQKIQLVNTNGS	83.3	1
	C2	----------A----------	16.7	
D	D1	KVLIVMLLFAGVDGVTHTSGAAAGHNARSLSGLFSLGSAQKLQLINTNGS	40	1
	D2	----------A-Y---GT--Y-TKTFT-F--R-PS--I--------	20	
	D3	----------T--Y---T-T----P--------V-----	10	
	D4	----------V---T----P--------V-----	10	
	D5	----------V-----	10	
	D6	----------Y-T--FT----S-----I--V-----	10	
E	E1	KVLIVMLLFAGVDGSTRVSGGQAGRVTKSLASFFSPGPQQKIQLVNSNGS	40	1
	E2	----------HGFT-L--A-S----------	30	
	E3	----------QGFT-L--A-S----------	10	
	E4	----------S-FT-L-TV----------	10	
	E5	----------N-Y---------AH--T-L--A-S----------	10	
F	F1	KVLIVMLLFAGVDGETNVMGGRAGHTTNTFTSLFSVGPAQKIQLVNSNGS	37	1
	F2	----------D-K----------S-L----N---S----------	27	
	F3	----------K----Q-----S-L----N---S----------	18	
	F4	----------A-------A--TK---------D----	9	
	F5	----------G--------A--A--L----TR--S----------	9	

consisting of 1 min at 94 °C, 2 min at 45 °C, and 3 min at 72 °C, were carried out, followed by a 10-min extension step at 72 °C. The second round was performed with the inner sense primer 5′-GGTAAGCTTTCCATGGTGGGGAACTGGGC-3′ (nt 1419–1447) and the inner antisense primer 5′-CTGGAATTCGCAGTCCTGTTGATGTGCCA-3′ (nt 1627–1599). The amplified products were cloned into the pGEM-T vector (Promega), and at least 8 independent clones were sequenced with an automatic DNA sequencer (ABI PRISM 310, PE Applied Biosystems).

3. Results

3.1. The outline of the RFB system

The RFB system was initially aimed at developing artificial liver tissues and allows liver-derived cells to maintain morphological appearance as well as their physiological functions, such as the ability to synthesize albumin and drug-metabolizing activity mediated by cytochrome P450 (Iwahori et al., 2003). Fig. 1 shows the outline of the RFB system. The bioreactor column consists of a vertically extended cylindrical matrix with porous glass microcarriers, which were most suitable for FLC4 culture as described in Section 2. The conditioning vessel is connected to a circulation system including tanks either for supplying fresh medium or for recovering sample aliquots. Oxygen consumption, temperature and pH of the culture medium are monitored continuously and conditioned in the vessel by computer and mass flow controller throughout the culture. Thus, the radial-flow configuration permits full contact between culture medium and cells at a physiologic perfusion flow rate, and prevents excessive shear stresses and a buildup of waste products, thus ensuring the long-term viability of 3D culture. For the long-term culture up to 110 days, temperature in the vessel gradually decreased from 37 to 30 °C as shown in Fig. 2A. The oxygen consumption, which indicates the cell growth condition, increased slowly from days 0 to 80 post-inoculation of the cells, and maintained a constant level afterwards. Under this condition, the production rate of albumin was found to be stable from days 15 to 105. The following experiments of HCV infection were done in such a stable phase of the cell condition after 3 weeks of pre-culture. Cell grown in the RFB column reached confluence at the end of culture (day 110) since the cells were observed outside the matrix bed (Fig. 2B).

3.2. Infection of HCV-positive sera to RFB cultured FLC4 cells

Previously, HCV RNA could be detected in FLC4 cells grown in the RFB up to 4 weeks of culture following inoculation with an HCV carrier plasmid (Aizaki et al., 2003). Establishment of a long-term stable culture system of human liver-derived cells

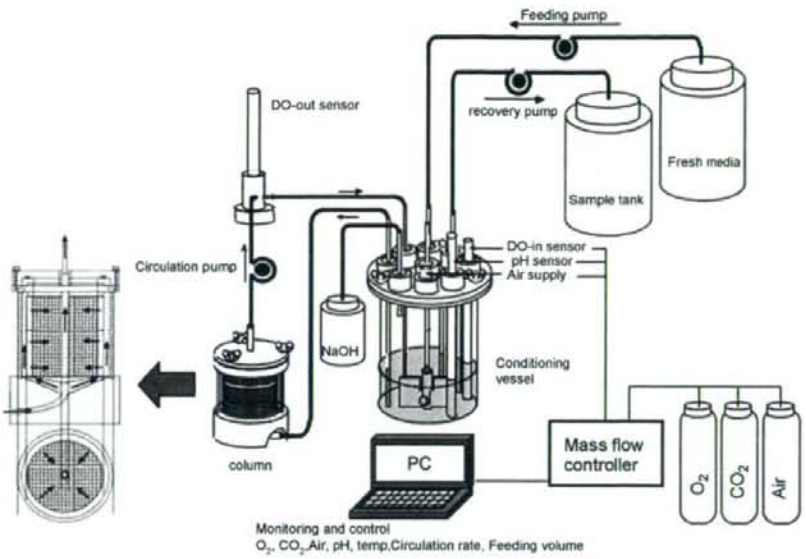


Fig. 1. Outline of the RFB system. RFB system consists of vessel, column and PC monitoring system. Culture condition was automatically controlled: oxgen concentration, temperature, pH, and oxgen level in the conditioning vessel are continuously monitored by PC and conditioned by mass flow controller.

retaining their differentiated hepatocyte function, as described above, enables evaluations of dynamic analysis of HCV replication and selection of viral variability and quasispecies. The potential of this culture system for screening HCV-positive sera was well suited for the viral infection.

Table 1 shows the serum samples (A–F) from six HCV carriers. The nucleotide complexity of HCV in serum samples was determined by sequencing the 1449–1598 nt region of the HCV genome, which includes HVR1 located at the N-terminal region of E2. Each serum was a mixture of a dominant HCV clone and related but distinct viral populations. The dominant species in sera A, C, D, E, and F were found to be genotype 1, and that in serum B was genotype 2. Viral loads in A–F, respectively, were 2.4×10^6, 8.6×10^6, 5.9×10^6, 2.5×10^6, 1.0×10^7 and 1.4×10^7 copies/mL, which were determined by real-time RT-PCR, as previously described (Aizaki et al., 2003; Suzuki et al., 2005). HCV loads of 2×10^6 copies from each serum sample were mixed to prepare a pooled serum sample containing 1.2×10^7 copies of HCV RNA. After FLC4 cells were inoculated into the RFB and subjected to 2 weeks of pre-culture for the preparation of 3D culture, the cells were infected with the pooled serum. Cell number at infection was about 10^8 in the 30-

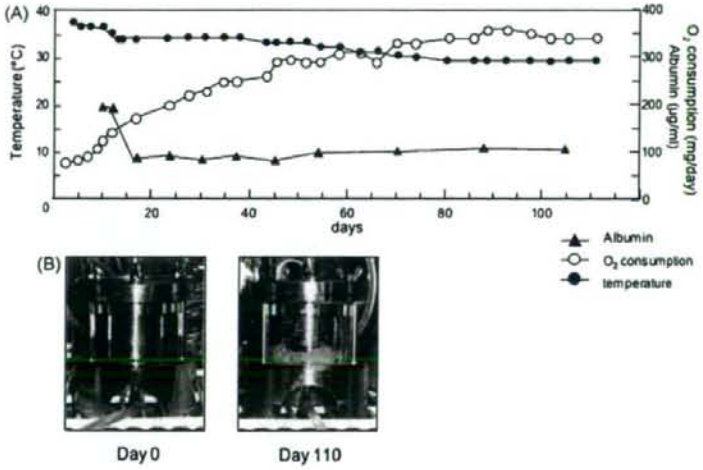


Fig. 2. Long-term culture of FLC4 cells in the RFB system. (A) Long-term culture of FLC4 cells in the RFB system. Temperature (closed circles) was gradually decreased from 37 to 30 °C. Oxygen consumption (open circles) was gradually increased from days 0 to 80 and reached the steady-state level. Albumin concentration (closed triangles) was constant from days 15 to 105. (B) The appearance of the RFB column at the beginning (day 0) and at the end (day 110) of culture.

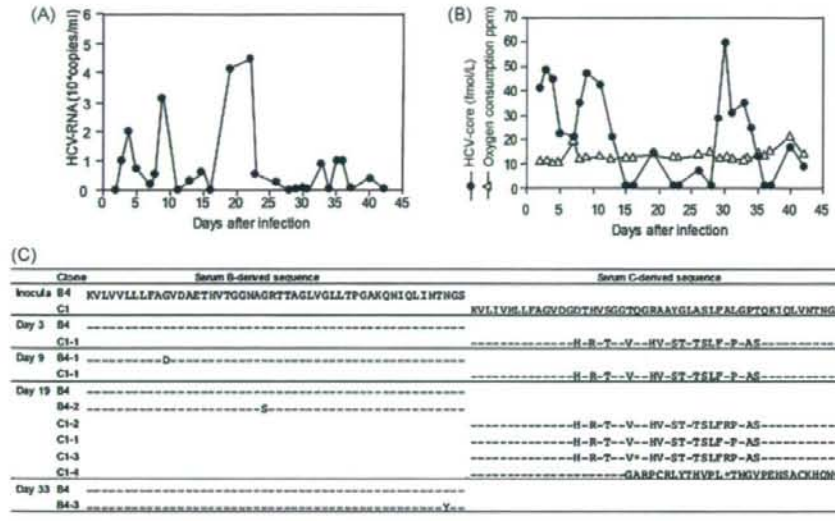


Fig. 3. HCV propagation in FLC4 cells cultured in the RFB system following inoculation with pooled sera obtained from HCV carriers. The 3D-cultured FLC4 cells were incubated with a pooled serum sample for 12 h, followed by changing the culture medium to fresh one. Culture medium was periodically collected for 42 days after inoculation, and HCV RNA and the viral core protein were quantified, respectively, by real-time RT-PCR and ELISA. (A) HCV RNA level in culture supernatant. (B) HCV-core protein (closed circles) and oxygen consumption (open triangles) levels in culture supernatant. (C) Changes in the viral quasispecies distribution after the inoculation. Percentages in the inoculum or in the culture medium at each time point (day 3, 9, 19, or 33 p.i.) are indicated at the right side. *, termination codon.

mL RFB column, as estimated from the glucose consumption (Kawada et al., 1998). Culture medium in the RFB was replaced with fresh medium 12 h post-infection (p.i.) and periodically sampled for 42 days.

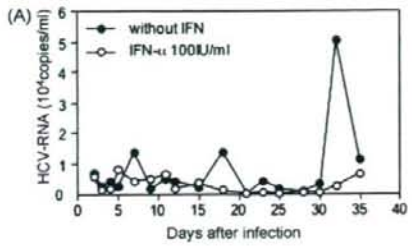
Fig. 3A and B shows the levels of HCV RNA and viral core protein in the culture medium, respectively. HCV RNA was not observed on the first 2 days following infection, but was detectable from day 3 p.i. Viral RNA levels fluctuated, with peaks on days 3, 9, 19–21 and 33–36 p.i. At days 19–21 p.i., the average amount of HCV RNA detected in the culture supernatant was approximately 3×10^6 copies/day. Intermittent peaks were observed in HCV core protein levels in the culture supernatant, and the peak pattern of the core protein was largely consistent with that of viral RNA. During the infection experiment, the level of oxygen consumption was constant at approximately 12 ppm, thus suggesting that the desired conditions (constant or very gradually increasing cell number) were maintained.

3.3. Quasispecies analysis in RFB culture

The above results suggest that, although the environment was consistent in the pooled serum infection, there were periods in which the viruses actively replicated and released from the cells and periods in which they poorly replicated. The pooled serum used for the infection exhibited HCV populations had at least 26 distinct quasispecies (Table 1). To investigate whether the quasispecies distribution was altered due to infection, and whether HCV populations are selected during long-term culture in the RFB, total RNA was extracted from the culture supernatant samples collected on days 3, 9, 19 and 33 p.i., and the nucleotide sequence of the region containing HVR1 was determined, as described above. As shown in Fig. 3C, it is of interest that only two HCV species were detected in the sample at day 3 p.i.; the dominant clone C1-1, comprising approximately 70% of the viral population, and clone B4, comprising 30%. Although clone C1-1 was not detected in the sequence of the inoculum shown in Table 1, it was most similar to clone C1, a dominant clone in plasma C, among the HCV population observed in the inoculum; thus, it is possible that clone C1-1 is one of the minor species in serum C. Clone B4 was found to be derived from serum B. An almost identical HCV population was observed in the sample at day 9 p.i. In this sample, the dominant clone C1-1 and clone B4-1, which differs from clone B4 by only one amino acid, were detected. In contrast, more significant variation in quasispecies structure of the HCV species was observed in the sample at day 19 p.i. than that at day 9 p.i. With B4 as the dominant clone, the serum B-derived HCV species, clones B4 and B4-2, which differs from clone B4 by one amino acid, comprised 58% of the total population. Four types of HCV sequences derived from serum C were detected. Two of these (clones C1-3 and C1-4) contained lethal mutations. It was also found that the HCV species detected in the sample at day 33 p.i. included only two clones (clones B4 and B4-3), derived from serum B. The dominant clone, B4, was found to comprise 89% of the total population.

3.4. Potential use of the RFB system for evaluation of anti-HCV compounds

An experiment was carried out to determine whether this HCV infection experiment system was useful for the evaluation of anti-HCV drugs (Fig. 4). For this purpose, a small,



(B)

	Clone		(%)
Inocula	B4	KVLVVLLLFAGVDAETHVTGGSAGRTTAGLVGLLTPGAKQNIQLINTNGS	2.1
	C1	KVLIVHLLFAGVDGDTHVSGGTQGRAATGLASLFALGPTQKIQLVNTNGS	13.9
Day 32	B4	----------	60
	B4-4	----------+----------	20
	C1-1	----------H-R-T--T--H--ST-TSLF-P-AS----------	20

Fig. 4. A therapeutic effect of IFN in HCV infection model in the RFB cultures. HCV-infected FLC4 cells were treated with or without 100 IU/mL IFN-α. (A) Culture media were periodically collected, and HCV RNA levels were determined. Closed circles: without IFN treatment, open circles: treatment with IFN. (B) Changes in the viral quasispecies distribution in the cells without IFN treatment. Percentages in the inoculum or in the culture medium on day 32 p.i. are indicated at the right side. *, termination codon.

4-mL RFB column was adopted and a pair of RFB cultures infected with the HCV-positive pooled plasma (Table 1) was prepared. IFN-α was added to one culture at a final concentration of 100 IU/mL at 12 h p.i. No cytotoxicity was observed in FLC4 cells under these conditions (data not shown). Culture media from two cultures (12.5 mL each) were sampled periodically for 35 days and replaced by the same volume of fresh medium in the presence or absence of IFN-α. HCV RNA in the collected media was quantified by real-time RT-PCR, as described above. As shown in Fig. 4A, in the no-treatment culture, fluctuations in the viral RNA levels with the peaks on days 7, 18, and 32 p.i. ($1.5–5 \times 10^4$ copies/mL) were observed. However, while HCV RNA at $0.5–0.8 \times 10^4$ copies/mL was detected in the IFN-treated culture at days 5–11 p.i., no HCV RNA was detected at days 12–30 p.i. Serum levels of hepatic transaminases such as ALT and AST are known to be markers of liver damage. In the HCV-infection model with FLC4 cells cultured in RFB, the AST levels in the culture medium, which ranged from 5 to 10 IU/L without HCV infection, increased to 20–50 IU/L according to the viral infection (data not shown). Such increased AST levels were found to fall by the IFN treatment to lower than 10 IU/L at day 28 p.i. As reported previously, the ALT levels in the culture medium were constantly low; its levels were less than 10 IU/mL, with or without HCV infection (Aizaki et al., 2003). The viral nucleotide sequence in the no-treatment culture medium at day 32 p.i. was determined. It was found that serum B-derived clone B4 was dominant, and serum C-derived clone C1 was present as a minor clone (Fig. 4B); thus, the results corresponded well with those demonstrated in Fig. 3. An increase in viral RNA in the IFN-treated culture after day 32 p.i. was observed; although the degree of increase was only slight (Fig. 4A). It will be interesting to test whether HCV species grown in the IFN-treated culture is a variant resistant to IFN-α.

4. Discussion

At present an important limitation of the *in vitro* HCV infection system is that the only established culture system is based on genotype 2a, JFH-1 isolate, and Huh-7-derived cell lines. The development of alternate infection systems in which other HCV strains and host cells are available has been needed for the study of HCV dynamics and virus–host interactions, and for testing antivirals. This paper demonstrates that a long-term culture of the 3D RFB system is a useful tool for investigating HCV dynamics. The present results revealed that the viral quasispecies distribution altered in the HCV infection system in the RFB system. The change probably occurs in the following two-stage process. The first change was observed on day 3 p.i.; thus, it is possible that the HCV species were selected according to infectivity in FLC4 cells. It has been reported that HCV particle populations in chronic hepatitis C patients consist of low-density virions and higher-density immune complex forms (Hijikata et al., 1993; Kanto et al., 1994). Inoculation of cultured cells with HCV has demonstrated that the immune complex forms were less infective than the antibody-unbound virions (Shimizu et al., 1994). Therefore, another hypothesis may be that a large number of HCV populations in sera A, D, E, and F are immune complex forms; thus, these sera are less susceptible to the cells than sera B and C. The second change was observed on days 19–33 p.i. While the serum C-derived clone was dominant in the early stages after infection, the serum B-derived HCV clone became dominant over time. In the absence of immunological selection pressure, viral nucleotide mutations at random positions are accumulated during viral replication, and the newly generated variant species are selected principally, if not solely, based on the intrinsic replicative advantages or disadvantages that these mutations confer. Thus, these results suggest that the use of pooled serum sample allowed for screening of infectious materials compatible for the RFB culture.

Evaluation methods for anti-HCV drugs using monolayer culture systems with various culture cells, such as the replicon system and the JFH-1 based virion production system, have been reported (Bartenschlager et al., 2003; Blight et al., 2000; Boriskin et al., 2006; Lanford et al., 2003; Lindenbach et al., 2005; Lohmann et al., 1999; Wakita et al., 2005; Zhong et al., 2005). These methods utilize viral markers, such as HCV RNA and antigens, as indicators of treatment efficacy. However, the utility of long-term cell culture systems for anti-HCV drug evaluation based on infection with human sera is still limited. The use of a chimpanzee model, the only non-human host for HCV infection, is restricted due to several reasons such as problematic availability and ethical consideration. Given intensive efforts to reduce and replace animal testing in the course of development of new therapies worldwide, the RFB-based HCV infection model is a potential alternative to animal models such chimpanzee for assessing anti-HCV compounds. According to the studies with regards to mathematical modeling of HCV kinetics (Dahari et al., 2005; Dixit et al., 2004; Layden et al., 2003; Layden-Almer et al., 2006; Perelson et al., 2005), IFN therapy against HCV infection generally generates a biphasic decline in viral load; there is a rapid decrease in the serum HCV RNA level over the

first 1 day of treatment, followed by the second phase, which is slower than the first-phase viral decline. To date, there were no such observable viral kinetics in the IFN treatment under such experimental settings. Further detailed kinetic analyses of the use of varying doses of IFN and of very early time points to evaluate the antiviral effect are in progress.

In summary, by investigating the dynamics of HCV populations in the RFB culture system, it was demonstrated that HCV was intermittently detected in the culture supernatants of long-term culture, and that changes in viral quasispecies appear to be related to this fluctuation in the virus level. It was also shown that an HCV-infection model using the RFB system is useful for evaluating potential antivirals. Further investigation on the infection and growth of various HCV-positive sera is currently being conducted in order to obtain an adaptive clone with higher replication efficiency in this culture system.

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Substitution of Amino Acid 70 in the Hepatitis C Virus Core Region of Genotype 1b Is an Important Predictor of Elevated Alpha-Fetoprotein in Patients Without Hepatocellular Carcinoma

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Previous studies identified amino acid (aa) substitutions of the hepatitis C virus core region of genotype 1b (HCV-1b core region) and elevated serum alpha-fetoprotein (AFP) levels as predictors of poor virologic response to pegylated interferon (PEG-IFN) plus ribavirin (RBV), and also as risk factors for hepatocarcinogenesis. The present study evaluated the impact of aa substitutions of HCV-1b core region on AFP, as a surrogate marker of hepatocarcinogenesis, on AFP levels in 569 Japanese patients with HCV-1b but without HCC, and investigated the predictive factors of elevated AFP ($\geq$11 μg/L). High AFP levels were detected in 27.4% of the patients. The rate of hepatocarcinogenesis in a group of 109 patients who received IFN monotherapy and followed-up for 15 years, was significantly higher in patients with abnormal than normal AFP. Multivariate analysis of 569 patients identified fibrosis stage (*F*3,4), aspartate aminotransferase ($\geq$76 IU/L), substitution of aa 70 (glutamine or histidine), and platelet count ($<15.0 \times 10^4$/μl) as significant determinants of elevated AFP. In 49 patients with abnormal AFP levels and substitutions at aa 70 who were treated with PEG-IFN + RBV, the rate of normalization of AFP was significantly lower in non-virological responders (28.6%) than in transient (71.4%) and sustained (100%) virological responders. The results indicated that substitution of aa 70 of HCV-1b core region is an important predictor of elevated AFP in non-HCC patients, and that eradication of the mutant virus normalizes AFP. The results highlight the importance of eradication of mutant type virus of aa 70 for reducing the risk of hepatocarcinogenesis. ***J. Med. Virol. 80:1354–1362, 2008.***

KEY WORDS: HCV; core region; genotype; AFP; hepatocellular carcinoma; glutamine; histidine

INTRODUCTION

Hepatitis C virus (HCV) usually causes chronic infection that can result in chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [Dusheiko, 1998; Ikeda et al., 1998; Niederau et al., 1998; Kenny-Walsh, 1999; Akuta et al., 2001]. In patients with HCV-chronic hepatitis, treatment with interferon (IFN) can induce viral clearance and marked biochemical and histological improvement [Davis et al., 1989; Di Bisceglie et al., 1989]. Especially, pegylated interferon (PEG-IFN) plus ribavirin (RBV) combination therapy can achieve a high sustained virological response, although patients with non-virological response who remain HCV-RNA-positive at the completion of treatment are also encountered [Akuta et al., 2005, 2006, 2007a,b,c]. Previous studies indicated that amino acid (aa) substitutions at position 70 and/or 91 in the HCV core region of genotype 1b (HCV-1b core region) and elevated alpha-fetoprotein (AFP) levels were predictors of poor virological response to PEG-IFN plus RBV therapy [Akuta et al., 2005, 2006, 2007a,b,c; Donlin et al., 2007], and also risk factors and surrogate markers of hepatocarcinogenesis [Ikeda et al., 2006; Akuta et al., 2007d].

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The use of elevated AFP as a predictor of early hepatocarcinogenesis in non-HCC patients might be clinically useful. AFP is a fetal glycoprotein produced by the yolk sac and fetal liver [Bergstrand and Czar, 1956], and has been used widely as a serum marker for the diagnosis of HCC [Sato et al., 1993; Johnson, 2001]. Furthermore, elevated serum AFP is also associated with various chronic liver diseases and hepatic regeneration [Kew et al., 1973; Silver et al., 1974; Elftherious et al., 1977; Alpert and Feller, 1978]. Although a mild rise in serum AFP is commonly seen in chronic HCV-infected patients, its clinicopathological significance remains to be defined. Previous studies indicated that high serum AFP levels correlated with fibrosis stages 3 and 4 [Bayati et al., 1998; Chu et al., 2001; Hu et al., 2002, 2004], levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) [Chu et al., 2001; Stein and Myaing, 2002; Hu et al., 2004], prothrombin time [Hu et al., 2004], and HCV-1b [Chu et al., 2001], in chronic HCV-infected patients. However, it is not clear whether mild elevation of AFP in the absence of HCC is associated with eventual development of HCC in HCV-infected patients. Furthermore, the impact of viral factors, such as aa substitutions of HCV-1b core region, on elevated AFP is still unclear.

The aims of the present study conducted in HCC-free Japanese patients infected with HCV-1b, were the following. (1) To evaluate the impact of elevated AFP, especially mild elevation of AFP, on hepatocarcinogenesis in IFN-treated patients without HCC during a long-term (15 years) follow-up period. (2) To identify the impact of aa substitutions in the core region on AFP levels in such patients, and determine the predictive factors for elevated AFP. (3) To investigate the normalization rates of AFP levels after eradication of HCV-RNA by PEG-IFN plus RBV combination therapy.

PATIENTS AND METHODS

Study Population

At Toranomon Hospital, Tokyo, Japan, 2,841 HCV-infected Japanese patients were recruited consecutively into the study protocol of IFN monotherapy between February 1987 and August 2007, and 929 HCV-infected Japanese patients were consecutively recruited into the study protocol of the combination therapy with PEG-IFNα-2b plus RBV between December 2001 and August 2007. Among these, 569 patients were selected in the present retrospective study based on the following criteria. (1) They were negative for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo), positive for anti-HCV (third-generation enzyme immunoassay, Chiron Corp., Emerville, CA), and positive for HCV-RNA qualitative analysis with PCR (nested PCR or Amplicor™, Roche Diagnostics, Indianapolis, IN). (2) They were naive to antiviral treatment. (3) They were infected with HCV-1b alone. (4) AFP levels were measured frequently, and substitutions of aa 70 or 91 in the HCV core region (HCV mutant-70 and HCV mutant-91, respectively) were determined at the commencement of the first course of antiviral treatment. (5) They were free of HCC based on clinical examination, laboratory tests, and imaging studies at baseline. (6) None was an alcoholic; lifetime cumulative alcohol intake was <500 kg (mild to moderate alcohol intake). (7) All were free of coinfection with human immunodeficiency virus. (8) None had other forms of hepatitis, such as hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease. (9) Each signed a consent form of the study protocol that had been approved by the Human Ethics Review Committee of Toranomon Hospital. Table I summarizes the profiles and laboratory data of the 569 patients at the commencement of antiviral treatment. They included 347 males and 222 females, aged 18–77 years (median, 55 years). Of the total group of 569 patients, 229 received IFN monotherapy, while 340 were treated with PEG-IFN plus RBV combination therapy. Among the patients who received IFN monotherapy, 109 patients started the monotherapy between February 1987 and August 1992, received at least two courses of such therapy, and were followed-up for 15 years. They were evaluated for the rate of development of HCC, associated with a rise in AFP level relative to that measured before the first course IFN monotherapy (baseline). At baseline, the latter group consisted of 80 males and 29 females, aged 22–69 with a median age of 46 years. The numbers of patients with fibrosis stages 1, 2, 3, and 4 were 57, 37, 14, and 1, respectively. The median AST and ALT levels were 85 IU/L (range, 27–400 IU/L) and 138 IU/L (range, 50–594 IU/L), respectively. The median platelet count was $17.0 \times 10^4/\mu l$ (range, 9.8×10^4 to $31.2 \times 10^4/\mu l$). The median viremia level was 5.8 Mequiv./ml (range, <0.5–46.5 Mequiv./ml). The median AFP level was 5 μg/L (range, 2–239 μg/L). The median follow-up time was 16.0 years (range, 0.1–20.3 years). With regard to

TABLE I. Profile and Laboratory Data of 569 Patients Infected with HCV Genotype 1b

Number of patients	569
Sex (male/female)	347/222
Age (years)*	55 (18–77)
Serum aspartate aminotransferase (IU/L)*	59 (17–400)
Serum alanine aminotransferase (IU/L)*	84 (15–594)
Platelet count ($\times 10^4/\mu l$)*	16.1 (3.8–40.2)
Serum alpha-fetoprotein (μg/L)*	6 (2–459)
Fibrosis stage (F1/F2/F3/F4/ND)	227/132/76/17/117
Level of viremia (high titer/low titer)**	522/47
Amino acid substitutions in core region***	
aa 70 (wild/mutant)	340/229
aa 91 (wild/mutant)	341/228
Treatment	
IFN monotherapy/PEG-IFN plus RBV	229/340

Data are number of patients, except those denoted by *, which represent the median (range) values. (**) Level of viremia was evaluated as high titer (≥1.0 Meq/ml, or ≥100 KIU/ml) and low titer (<1.0 Meq/ml, or <100 KIU/ml). (***) The presence of arginine at aa 70 was evaluated as wild type, while other patterns (glutamine/histidine) as mutant type. The presence of leucine at aa 91 was evaluated as wild type, while other patterns (methionine) as mutant type.
Normal reference ranges: 11–38 IU/L for aspartate aminotransferase; 6–50 IU/L for alanine aminotransferase (IU/L); ≤10 μg/L for alpha-fetoprotein. ND: not done; IFN: interferon; PEG-IFN: pegylated interferon; RBV: ribavirin.

the protocol of IFN monotherapy, 68 (62.4%) patients received IFN-α alone; 36 (33.0%) patients received IFN-β alone; while the remaining 5 (4.6%) patients received a combination of IFN-α and IFN-β. The median IFN dose per day of 6 million units (MU, range; 1–10 MU) was administered. IFN monotherapy included initial aggressive induction therapy, consisting of every day within the first 8 weeks of commencement of therapy, followed subsequently by three times per week.

On the other hand, 340 patients received PEG-IFNα-2b combination therapy at a median dose of 1.5 μg/kg (range, 0.8–1.8 μg/kg) subcutaneously each week plus oral RBV at a median dose of 11.0 mg/kg (range, 3.4–14.2 mg/kg) daily for a median duration of 48 weeks (range, 9–112 weeks).

In this study, patients who were HCV-RNA-negative by qualitative PCR analysis at 24 weeks after the completion of therapy, were defined as sustained virological responders. On the other hand, patients who were HCV-RNA-negative by qualitative PCR analysis at the completion of 24-week treatment but became HCV-RNA-positive after the 24-week therapy, were defined as transient virological responders. Patients who remained HCV-RNA-positive by quantitative and/or qualitative PCR analyses at the completion and after treatment, were defined as non-virological responders.

Laboratory Investigations

Blood samples were obtained at least once every month before, during, and after treatment, and were analyzed for AST, ALT, and HCV-RNA levels. The serum samples were frozen at −80°C within 4 hr of collection and then thawed at the time of measurement. HCV genotype was determined by PCR using a mixed primer set derived from nucleotide sequences of NS5 region [Chayama et al., 1993]. HCV-RNA levels were measured by branched DNA assay version 2.0 (Chiron Corp., Emeryville, CA) or quantitative PCR assay (Cobas Amplicor HCV monitor v 2.0 using the 10-fold dilution method, Roche) before, during, and after the antiviral therapy. The lower limits of these assays were 0.5 Meq/ml (10^6 genomic equivalents per milliliter) by branched DNA assay, or 5 KIU/ml by quantitative PCR assay. Samples with undetectable levels by these quantitative assays (<0.5 Meq/ml, or <5 KIU/ml) were checked also by HCV-RNA qualitative analysis with PCR (nested PCR or Amplicor™, Roche) during and after treatment especially, and the results were expressed as positive or negative. The lower limit of the assay was 50 IU/ml. In this study, levels of viremia were evaluated as high titer (≥1.0 Meq/ml, or ≥100 KIU/ml) and low titer (<1.0 Meq/ml, or <100 KIU/ml).

Histopathological Examination of Liver Biopsies

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo). The biopsy material was fixed in 10% formalin, and stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid–Schiff after diastase digestion. All specimens for examination contained six or more portal areas. Histopathological diagnosis was confirmed by an experienced liver pathologist (H.K.) who was blinded to the clinical data. Chronic hepatitis and liver cirrhosis were diagnosed based on histological assessment according to the scoring system of Desmet et al. [1994].

Detection of Amino Acid Substitutions in Core Region

Okamoto et al. [2007] developed a simple PCR method for detecting substitutions of aa 70 or aa 91 in HCV-1b core region using mutation-specific primer, as an alternative to the direct sequencing method. The major protein type was determined based on the relative intensity of the bands for wild (aa 70: arginine, aa 91: leucine) and mutant HCV-1b (aa 70: glutamine/histidine, aa 91: methionine) in agarose gel electrophoresis. If the intensities of the bands were similar, the case was regarded as competitive. The detection rate was 94.4%, the sensitivity was 10 KIU/ml using quantitative assay with PCR (Cobas Amplicor HCV monitor v 2.0 using the 10-fold dilution method, Roche), the reproducibility was high, and consistency with direct sequencing was 97.1% in positive cases. Mutation in this study refers to substitution from consensus sequence. In previous studies, HCV-J (accession no. D90208) was considered a prototype and the aa substitution was evaluated by comparison with the consensus sequence prepared from 50 clinical trial samples [Kato et al., 1990; Akuta et al., 2005]. In the present study, PCR using primers specific for substitutions of aa 70 or aa 91 was performed in samples collected from 454 patients [Okamoto et al., 2007]; the remaining 115 patients were analyzed by direct sequencing [Akuta et al., 2005, 2006].

Diagnosis of Hepatocellular Carcinoma

Patients were examined for HCC by abdominal ultrasonography every 3–6 months. If HCC was suspected based on ultrasonographic results, additional procedures, such as computed tomography, magnetic resonance imaging, abdominal angiography, and ultrasonography-guided tumor biopsy, were used to confirm the diagnosis.

Statistical Analysis

Non-parametric tests were used to compare variables between groups, including the Mann–Whitney *U*-test, chi-squared test and Fisher's exact probability test. Multiple comparisons were conducted by the Bonferroni test. The cumulative rate of hepatocarcinogenesis was calculated using the Kaplan–Meier technique; differences between carcinogenesis curves between groups were tested using the log-rank test. Statistical analyses of the rate of hepatocarcinogenesis according to

groups were calculated using the period from start of the first course of IFN monotherapy. Univariate and multivariate logistic regression analyses were used to determine the independent predictive factors of elevated AFP. The odds ratios and 95% confidence intervals (95% CI) were also calculated. All P values less than 0.05 by the two-tailed test were considered significant. Variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. Potential predictive factors associated with elevated AFP included the following pretreatment variables: sex, age, AST, ALT, platelets, pathological staging, viremia level, and aa substitutions in the core region. Statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL).

RESULTS

Cumulative Rate of Hepatocarcinogenesis According to AFP Levels

Of the 229 patients who received IFN monotherapy, 109 could be evaluated for the rate of development of HCC based on AFP levels measured at the start of the first course IFN monotherapy (baseline), during a follow-up period of 15 years. All 109 patients received two or more courses of IFN monotherapy; 66 patients received two courses of IFN (including 16 patients who achieved sustained virological response), 35 patients received three courses (including 4 patients who achieved sustained virological response), 7 patients received four courses (including 1 patient who achieved sustained virological response), and one patient received six courses (did not achieve sustained virological response). Thus, 21 of 109 patients achieved sustained virological response after multicourses of IFN monotherapy. For those who received 1, 2, 3, 4, 5, and 6 courses of IFN monotherapy, the median total duration of IFN therapy was 23.9 weeks (range, 0.9–134.7 weeks), 24.0 (range, 1.3–313.7), 25.1 (range, 3.1–193.1), 40.3 (range, 21.0–86.3), 23.6, and 67.9, respectively, and the median total dose of IFN was 526 MU (range, 22–1393 MU), 589 (range, 57–4005), 501 (range, 28–3477), 536 (range, 363–1553), 708, and 1200, respectively. The median cumulative total duration and cumulative total dose, which represented the cumulative total duration and total dose of every course of every patient were 57.7 weeks (range, 14.0–467.6 weeks) and 1380 MU (range, 521–4805 MU), respectively. The median period during which no IFN was administered was 3.7 years (range, 0.1–7.0 years). Finally, the median dose of IFN per week was 22.5 MU (range, 3.7–43.9).

During the follow-up, 8.6% (7 of 81 patients), 20.0% (3 of 15), and 38.5% (5 of 13) developed HCC in patients with AFP levels below 1 (≤10 μg/L), from 1 to 2 (11–20 μg/L), and above twice (≥21 μg/L) the upper limit of normal (ULN), respectively. In patients with AFP levels below 1, from 1 to 2, and above 2 times the ULN, the cumulative hepatocarcinogenesis rates were 0, 7.1, 0% at the end of 5 years; 3.1, 23.4, 37.5% at the end of 10 years; and 14.5, 23.4, 58.3% at the end of 15 years, respectively. The rates were significantly different among the three groups ($P < 0.001$; log-rank test) (Fig. 1). Especially, the rate of hepatocarcinogenesis in patients with normal AFP levels was significantly lower than in those with AFP levels above twice ULN ($P < 0.001$), and tended to be lower than in those with AFP levels from 1 to 2 times ULN ($P = 0.070$). The rate of hepatocarcinogenesis in patients with AFP levels above twice ULN was not significantly higher than in those with AFP levels from 1 to 2 times ULN. Thus, the rate of hepatocarcinogenesis was significantly higher in patients with abnormal AFP levels than in those with normal AFP levels ($P < 0.001$).

Predictive Factors of Elevated AFP in Univariate and Multivariate Analyses

The virological, clinical, and biochemical features of the whole population sample of 569 patients were analyzed to determine factors that could predict elevated AFP (≥11 μg/L). Elevated AFP was detected in 156 of 569 (27.4%) patients. Univariate analysis identified seven parameters that influenced significantly high AFP level. These included age (≥45 years, $P = 0.001$), AST (≥76 IU/L, $P < 0.001$), ALT (≥100 IU/L, $P < 0.001$), platelets ($<15.0 \times 10^4/\mu l$, $P < 0.001$), stage of fibrosis ($F3,4$, $P < 0.001$), and aa substitutions of the core region (mutant type of aa 70, $P < 0.001$, and aa 91, $P = 0.035$). Multivariate analysis identified four parameters that independently influenced high AFP level, including stage of fibrosis ($F3,4$, $P < 0.001$), AST (≥76 IU/L, $P < 0.001$), substitution of aa 70 (mutant type, $P < 0.001$), and platelet count ($<15.0 \times 10^4/\mu l$, $P = 0.019$) (Table IIA).

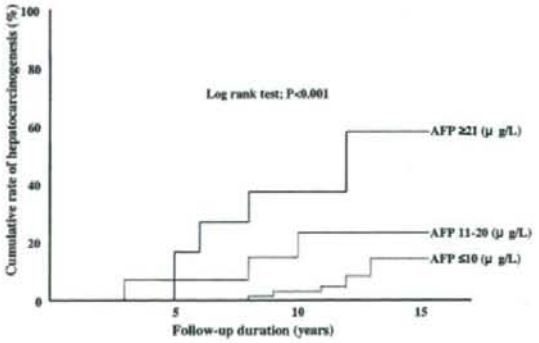

Fig. 1. Cumulative rate of hepatocarcinogenesis according to AFP levels at the start of first course IFN monotherapy. The rate of hepatocarcinogenesis in patients with normal AFP levels (≤10 μg/L) was significantly lower than in those with AFP levels above twice the upper limit of normal (≥21 μg/L) ($P < 0.001$), and tended to be lower than in those with AFP levels from 1 to 2 times the upper limit of normal (11–20 μg/L) ($P = 0.070$). The rate of hepatocarcinogenesis in patients with abnormal AFP levels (≥11 μg/L) was significantly higher than in those with normal AFP levels ($P < 0.001$).

TABLE IIA. Factors Associated with Elevated Serum AFP Levels (≥11 μg/L) in Patients Infected with HCV Genotype 1b, Identified by Multivariate Analysis

Factor	Category	Odds ratio (95% CI)	P
Fibrosis stage	1: F1,2	1	
	2: F3,4	5.014 (2.746–9.153)	<0.001
Aspartate aminotransferase (IU/L)	1: <76	1	
	2: ≥76	4.592 (2.707–7.789)	<0.001
Substitution of aa 70	1: wild type	1	
	2: mutant type	2.618 (1.561–4.391)	<0.001
Platelet count ($\times 10^4/\mu l$)	1: ≥15.0	1	
	2: <15.0	1.912 (1.111–3.289)	0.019

*The presence of arginine at aa 70 was evaluated as wild type, while other patterns (glutamine/histidine) as mutant type. Normal reference ranges: ≤10 μg/L for alpha-fetoprotein.

The entire population sample was also analyzed to determine factors that could predict elevated AFP above twice ULN (≥21 μg/L); which was noted in 75 of 569 (13.2%) patients. Univariate analysis identified seven parameters that significantly influenced elevated AFP above twice ULN. These included age (≥45 years, $P = 0.015$), AST (≥76 IU/L, $P < 0.001$), ALT (≥100 IU/L, $P < 0.001$), platelet count ($<15.0 \times 10^4/\mu l$, $P < 0.001$), stage of fibrosis (F3,4, $P < 0.001$), and aa substitutions of the core region (mutant type of aa 70, $P < 0.001$, and aa 91, $P = 0.008$). Multivariate analysis identified four parameters that influenced independently elevated AFP above twice ULN, including stage of fibrosis (F3,4, $P < 0.001$), AST (≥76 IU/L, $P < 0.001$), and aa substitutions of the core region (HCV mutant-91, $P = 0.029$, and -70, $P = 0.056$) (Table IIB).

AFP Levels and aa Substitutions of Core Region

The entire population sample was also analyzed to determine the relationship between aa substitutions of the core region and AFP levels. The proportions of patients with HCV mutant-70 among those with AFP levels below 1, from 1 to 2, from 2 to 4, from 4 to 8, and above 8 times ULN were 33.4% (138 of 413 patients), 53.1% (43 of 81), 60.0% (24 of 40), 66.7% (8 of 12), and 69.6% (16 of 23) (Fig. 2A). Thus, the higher the proportion of patients with HCV mutant-70, the higher the AFP level, and significantly lower proportions of patients with HCV mutant-70 were noted among those with normal AFP levels (33.4%) than those with AFP levels from 1 to 2 times (53.1%) ($P = 0.001$) and above twice ULN (64.0%) ($P < 0.001$).

The proportions of patients with HCV mutant-91 among those with AFP levels below 1, from 1 to 2, from 2 to 4, from 4 to 8, and above 8 times ULN were 37.3% (154 of 413 patients), 40.7% (33 of 81), 67.5% (27 of 40), 25.0% (3 of 12), and 47.8% (11 of 23) (Fig. 2B). Thus, a higher frequency of HCV mutant-91 did not correlate with high AFP levels. In particular, significantly higher proportion of patients with HCV mutant-91 were noted among those with AFP levels from 2 to 4 times ULN (67.5%) than in those with AFP levels below 2 times (37.9%, $P < 0.001$) and above 4 times (40.0%, $P = 0.021$).

Normalization Rates of AFP Levels Based on Eradication of HCV-RNA With PEG-IFN Plus RBV Combination Therapy

Finally, the proportion of patients who showed normalization of AFP after commencement of PEG-IFNα-2b plus RBV combination therapy was determined in those at high risk for hepatocarcinogenesis, who had abnormal AFP levels (>10 IU/L) and HCV mutant-70 at baseline. Of the 340 patients, 49 had both abnormal AFP level and HCV mutant-70 at baseline. Of these, 14.3% (7 of 49 patients) could achieve sustained virological response, 28.6% (14 of 49) showed transient virological response, and 57.1% (28 of 49) had non-virological response. Table III summarizes the characteristics of

TABLE IIB. Factors Associated with Elevated Serum AFP Above Twice the Upper Limit of Normal (≥21 μg/L) in Patients Infected with HCV Genotype 1b, Identified by Multivariate Analysis

Factor	Category	Odds ratio (95% CI)	P
Fibrosis stage	1: F1,2	1	
	2: F3,4	6.875 (3.485–13.56)	<0.001
Aspartate aminotransferase (IU/L)	1: <76	1	
	2: ≥76	6.144 (3.088–12.23)	<0.001
Substitution of aa 91	1: wild type	1	
	2: mutant type	2.101 (1.077–4.099)	0.029
Substitution of aa 70	1: wild type	1	
	2: mutant type	1.914 (0.984–3.722)	0.056

*The presence of arginine at aa 70 was evaluated as wild type, and other patterns (glutamine/histidine) as mutant type. The presence of leucine at aa 91 was evaluated as wild type, and other pattern (methionine) as mutant type. Normal reference ranges: ≤10 μg/L for alpha-fetoprotein.

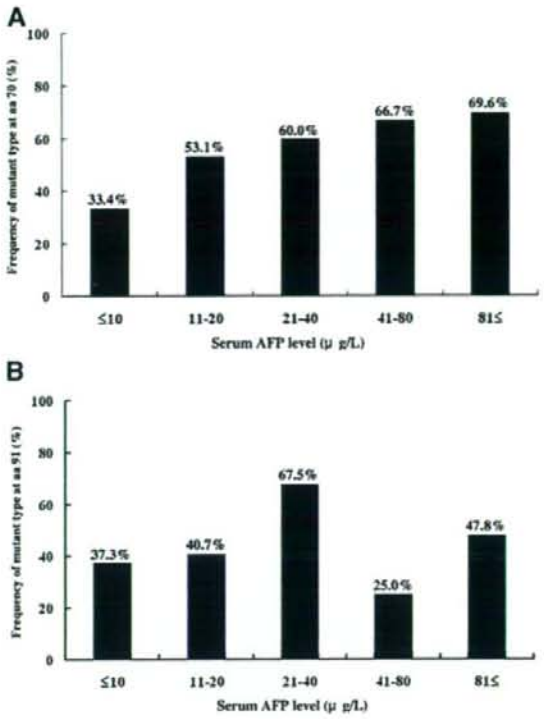

Fig. 2. **A**: Frequency of mutation in aa at position 70 of the HCV-1b core region according to serum AFP levels. Higher frequencies of the mutation correlated with higher serum AFP levels. Significantly lower frequencies of the mutant type were noted in patients with normal AFP levels (≤10 μg/L) than in those with levels from 1 to 2 times (11–20 μg/L, $P = 0.001$) and above twice the upper limit of normal (≥21 μg/L, $P < 0.001$), respectively. **B**: Frequency of mutation in aa at position 91 of the HCV-1b core region according to serum AFP levels. Higher frequencies of the mutation did not correlate with higher AFP levels. Significantly higher frequencies of the mutant type were noted in patients with AFP levels from 2 to 4 times the upper limit of normal (21–40 μg/L) than in those with levels below 2 times (≤20 μg/L, $P < 0.001$) and above 4 times (≥41 μg/L, $P = 0.021$).

these 49 patients at the commencement of combination therapy, according to treatment efficacy. The duration of treatment of non-virological responders was significantly shorter than that of sustained- ($P < 0.001$; Bonferroni test) and transient-virological responders ($P = 0.011$; Bonferroni test). Furthermore, AST levels of non-virological responders were significantly lower than those of sustained virological responders ($P = 0.049$; Bonferroni test). However, there were no significant differences in other patient characteristics at the commencement of treatment among the three groups.

The proportions of patients who showed normalization of AFP at the completion of treatment were 71.4% (5 of 7), 71.4% (10 of 14), and 53.6% (15 of 28) for the sustained-, transient-, and non-virological responders, respectively. There were no significant differences in the normalization rates at the completion of treatment among the three groups (Bonferroni test). However, the proportions of patients who showed normalization of AFP at 24 weeks after completion of treatment were 100% (7 of 7), 71.4% (10 of 14), and 28.6% (8 of 28) in the sustained-, transient-, and non-virological responders, respectively. The normalization rate in non-virological responders was significantly lower than in sustained- ($P = 0.001$; Bonferroni test) and transient virological responders ($P = 0.012$; Bonferroni test) (Fig. 3).

DISCUSSION

Elevated AFP in HCV-infected patients without HCC might be useful early predictor of hepatocarcinogenesis, but there is little evidence that mild elevation of AFP in such patients is associated with eventual development of HCC. Ikeda et al. [2006] reported that AFP level above twice ULN was an independent and significant determinant of hepatocarcinogenesis in patients with HCV-related cirrhosis. The present study of HCV-infected patients treated with IFN and followed for up to 15 years also showed that the rate of hepatocarcinogenesis was significantly higher in patients with abnormal AFP levels than in those with normal levels. In particular, the rate of hepatocarcinogenesis in patients with normal AFP levels was significantly lower than in those with levels above twice the ULN, and tended to be lower than in those with levels from 1 to 2 times ULN (i.e., mild elevation of AFP). To our knowledge, the present study is the first to report the hepatocarcinogenesis rates according to AFP levels in HCV-infected patients followed over a 15-year period, including mild elevation of AFP in patients without HCC.

Despite numerous epidemiologic studies linking HCV infection and the development of HCC, it remains controversial whether HCV itself plays direct or indirect role in the pathogenesis of HCC [Koike, 2005]. Studies using transgenic mice concluded that the HCV core region can potentially cause HCC [Moriya et al., 1998], but the clinical impact of HCV core region on hepatocarcinogenesis is still not clear. Previous studies identified substitutions in aa 70 and/or 91 in the HCV-1b core region and elevated AFP levels as predictors of poor virological response to PEG-IFN plus RBV [Akuta et al., 2005, 2006, 2007a,b,c; Donlin et al., 2007], and also as risk factors for hepatocarcinogenesis [Ikeda et al., 2006; Akuta et al., 2007d]. It is speculated that cases resistant to treatment might ultimately develop HCC. The present study indicated that mutation in aa 70 in the core region predicted elevation of AFP in HCV-infected non-HCC patients. These results support the oncogenic potential of the HCV core region and clinically link mutations in this region to HCC.

Previous reports identified PA28γ-dependent pathway as a mechanism of HCV-associated hepatocarcinogenesis. Moriishi et al. demonstrated that knockout of the PA28γ gene induced accumulation of HCV core protein in nuclei of hepatocytes of HCV core gene transgenic mice and disrupted the development of both hepatic steatosis and HCC [Moriishi et al., 2003, 2007]. Furthermore, HCV core protein also enhanced the

TABLE III. Patient Characteristics at Commencement of Combination Therapy of Pegylated Interferon α-2b Plus Ribavirin, of 49 Patients with Abnormal AFP Levels and Mutant Type of aa 70

	SVR (n = 7)	TVR (n = 14)	NVR (n = 28)
Sex (male/female)	3/4	9/5	12/16
Age (years)*	58 (43–64)	56 (34–63)	57 (43–66)
Serum aspartate aminotransferase (IU/L)*	83 (37–324)[a]	84 (34–266)	76 (28–135)
Serum alanine aminotransferase (IU/L)*	99 (41–344)	126 (42–504)	82 (37–218)
Platelet count ($\times 10^4/\mu l$)*	11.6 (8.0–19.3)	14.1 (7.5–20.6)	12.4 (6.6–27.3)
Serum alpha-fetoprotein (μg/L)*	17 (11–161)	21 (11–38)	22 (11–427)
Fibrosis stage (F1/F2/F3/F4/ND)	0/3/2/0/2	2/0/5/0/7	6/3/7/2/10
Level of viremia (high titer/low titer)**	7/0	14/0	27/1
Amino acid substitutions in core region***			
aa 70 (wild/mutant)	0/7	0/14	0/28
aa 91 (wild/mutant)	5/2	6/8	16/12
Treatment duration (weeks)	75 (60–85)[b]	53 (46–77)[c]	47 (12–112)

Data are number of patients, except those denoted by *, which represent the median (range) values. (**) Level of viremia was evaluated as high titer (≥1.0 Meq/ml, or ≥100 KIU/ml) and low titer (<1.0 Meq/ml, or <100 KIU/ml). (***) The presence of arginine at aa 70 was evaluated as wild type, and other patterns (glutamine/histidine) as mutant type.
The presence of leucine at aa 91 was evaluated as wild type, and other pattern (methionine) as mutant type.
Normal reference ranges: 11–38 IU/L for aspartate aminotransferase; 6–50 IU/L for alanine aminotransferase (IU/L); ≤10 μg/L for alpha-fetoprotein.
SVR: sustained virological response; TVR: transient virological response; NVR: non-virological response; ND: not done. [a]$P = 0.049$, [b]$P < 0.001$, [c]$P = 0.011$, compared with NVR by Bonferroni test.

binding of liver X receptor α (LXRα)/retinoid X receptor α (RXRα) to the LXR-response element in the presence of PA28γ [Moriishi et al., 2007]. Thus, PA28γ could play a crucial role in the development of HCV-associated steatogenesis and hepatocarcinogenesis. Further studies are necessary to link the results of animal studies and the clinical impact of aa substitutions in HCV core region on hepatocarcinogenesis.

Chu et al. [2001] indicated that elevation of AFP in the absence of HCC might be associated with HCV-1b infection, and that such rise could correlate with more severe hepatic necroinflammation and fibrosis/cirrhosis and higher viremia levels. The results of the present study indicated that patients infected with HCV mutation-70 had elevated serum AFP levels, although the relation between HCV mutation-91 and AFP was not very clear. On the one hand, multivariate analysis identified HCV mutation-91 as an independent and significant determinant of elevated AFP levels above twice the ULN. On the other; however, a significantly higher proportion of patients infected with HCV mutant-91 had AFP levels from 2 to 4 times ULN compared to those with levels below 2 times and levels above 4 times, i.e., there was no relation between the frequency of HCV mutant-91 and serum AFP levels. Further large-scale studies should be performed to investigate the relationship between HCV mutant-91 and elevated AFP.

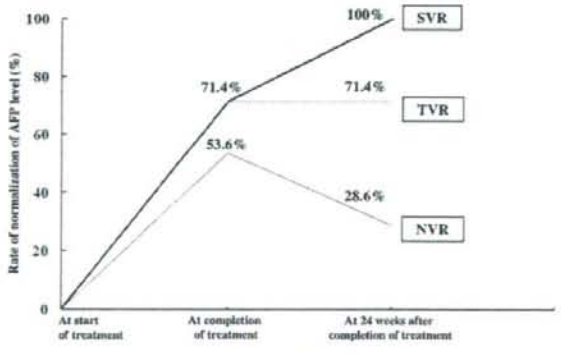

Fig. 3. Normalization rates of AFP levels at and 24 weeks after completion of treatment in sustained virological responders (SVR), transient virological responders (TVR), and non-virological responders (NVR).

Previous studies reported that IFN monotherapy [Arase et al., 2007] and IFN plus RBV combination therapy [Yu et al., 2006; Chen et al., 2007] results in reduction of AFP levels and the likelihood of hepatocarcinogenesis. In the present study, viral eradication (sustained virological response) in patients who received PEG-IFN plus RBV combination therapy was associated with normalization of AFP in patients at high risk for hepatocarcinogenesis (i.e., those with abnormal AFP levels and HCV mutant-70). These results emphasize that the risk of hepatocarcinogenesis could be reduced by eradication of HCV mutant-70. The results also showed that the proportion of patients with normalization of AFP levels was significantly higher in transient virological responders than in non-virological responders, suggesting that transient virological response could also result in the suppression of hepatocarcinogenesis, even when a sustained virological response is not achieved. In Japan, only 3 years had elapsed since the introduction of PEG-IFNα-2b plus RBV combination therapy into the Japanese Government Health Insurance system, and accordingly, the long-term effects of this combination therapy on hepatocarcinogenesis could not be evaluated in the present study. Further studies

that include patients treated with not only IFN but also PEG-IFN plus RBV, should be performed in the future.

In conclusion, the results of the present study indicated that substitution of aa at position 70 of the HCV-1b core region can predict elevation of serum AFP levels in non-HCC patients, and that eradication of the mutant virus seems to induce normalization of AFP. This finding highlights the importance of eradication of this mutant virus in reducing the risk of hepatocarcinogenesis. The limitations of the present study were that it did not investigate other genotypes apart from HCV-1b, the geographic diversities of HCV-1b core region (distribution of wild or mutant type), and the study of other races apart from Asians in Japan. Further prospective studies, matched for HCV genotype, aa substitutions of the core region, and race, of a large group of patients are required to determine the meaning of elevated AFP in non-HCC patients.

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Journal of Medical Virology 80:1363–1369 (2008)

Efficacy of Low-Dose Intermittent Interferon-Alpha Monotherapy in Patients Infected With Hepatitis C Virus Genotype 1b Who Were Predicted or Failed to Respond to Pegylated Interferon Plus Ribavirin Combination Therapy

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The efficacy of interferon (IFN) monotherapy for non-responders to pegylated interferon (PEG-IFN) plus ribavirin (RBV) combination therapy is still unclear. To evaluate the impact of IFN monotherapy on biochemical response, 200 consecutive patients infected with HCV genotype 1b, who received low-dose intermittent IFN-alpha monotherapy, were investigated. A median IFN dose per day of 3 million units was administered during a median period of 74 weeks. As a whole, the ALT normalization rates were 50.5, 65.9, 58.4, and 61.7% at 4, 12, 24, and 48 weeks, respectively. In 40 patients, who had abnormal AFP levels at the start of treatment, 52.5% achieved normalization of AFP within 48 weeks. Multivariate analysis identified indocyanine green retention rate at 15 min as the parameter that influenced significantly and independently ALT normalization. ALT normalization rates of patients who were predicted to be poor responders to PEG-IFN plus RBV combination therapy (but not substitutions of amino acid 70 and/or 91 in the HCV core region, female sex, and lower levels of low-density lipoprotein cholesterol) were similar to others. Furthermore, the ALT normalization rates in non-responders to combination therapy were 29.2, 60.9, 60.0, and 40.0% at 4, 12, 24, and 48 weeks, respectively. The results suggest that low-dose intermittent IFN monotherapy is an efficacious therapeutic regimen for patients unsuitable for PEG-IFN plus RBV, including non-responders, because it can lead to ALT normalization and thus a reduced risk of hepatocarcinogenesis. ***J. Med. Virol. 80:1363–1369, 2008.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: HCV; interferon; ribavirin; ALT; hepatocellular carcinoma; core region; AFP; low-density lipoprotein cholesterol

INTRODUCTION

Hepatitis C virus (HCV) usually causes chronic infection, which can result in chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [Dusheiko, 1998; Ikeda et al., 1998; Niederau et al., 1998; Kenny-Walsh, 1999; Akuta et al., 2001]. Treatment of HCV-chronic hepatitis with interferon (IFN) can induce viral clearance and marked biochemical and histological improvement [Davis et al., 1989; Di Bisceglie et al., 1989].

Pegylated interferon (PEG-IFN) plus ribavirin (RBV) combination therapy for chronic HCV infection is expensive and associated with severe side effects but treated patients show a high-sustained virological response. Patients who do not achieve sustained virological response need to be identified before the start of combination therapy, in order to avoid unnecessary side effects and high costs. Thus, the safer IFN monotherapy should be selected as the therapeutic regimen for patients unsuitable for PEG-IFN plus RBV therapy. In a series of papers, Akuta et al. [2005a, 2006, 2007a,b,c] studied determinants of the response to PEG-IFN plus RBV in patients with high titers of genotype 1b (≥100 kiloIU [KIU]/ml), which is

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dominant in Japan. They identified substitutions of amino acid (aa) 70 and/or 91 in the HCV core region, female sex, and low levels of low-density lipoprotein cholesterol as independent and significant pretreatment negative predictors associated with virological response. Furthermore, previous studies reported that low-dose intermittent IFN monotherapy, as a treatment strategy, induces biochemical response [i.e., normalization of alanine aminotransferase (ALT) and alpha-fetoprotein (AFP) levels] and reduces the risk of hepatocarcinogenesis, even if patients failed to achieve sustained virological response [Arase et al., 2001, 2007; Nomura et al., 2007; McHutchison et al., 2008]. Hence, low-dose intermittent IFN monotherapy might be beneficial therapeutically in reducing the risk of hepatocarcinogenesis in patients who are predicted to be non-responsive to PEG-IFN plus RBV.

The present study included 200 consecutive patients infected with HCV genotype 1b, who were treated by self-injection of low-dose intermittent natural IFN-alpha. The aims of the study were the following. (1) To investigate the normalization rates of alanine aminotransferase (ALT) and α-fetoprotein (AFP) levels within 48 weeks after the commencement of treatment. (2) To examine the predictive factors associated with ALT normalization. (3) To evaluate the efficacy of IFN monotherapy in patients with predictors of poor response to IFN plus RBV combination therapy. (4) To evaluate the efficacy of IFN monotherapy for non-responders to IFN plus RBV combination therapy.

PATIENTS AND METHODS

Patients

Among 252 consecutive HCV-infected patients who started IFN monotherapy between April 2005 and July 2007 at Toranomon Hospital, 200 were selected in the present study based on the following criteria. (1) Patients treated by self-injection of natural IFN-alpha (Sumiferon®; Sumitomo Pharmaceutical Co., Osaka, Japan). (2) Patients infected with HCV genotype 1b alone. (3) Patients negative for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan), positive for anti-HCV (third-generation enzyme immunoassay, Chiron Corp, Emerville, CA), and positive for HCV RNA qualitative analysis with PCR (Amplicor, Roche Diagnostic Systems, Pleasanton, CA). (4) Patients who have not been treated with antiviral or immunosuppressive agents, except for IFN plus RBV combination therapy, within 6 months of enrolment. (5) Patients free of HCC. (6) Patients free of coinfection with human immunodeficiency virus. (7) Lifetime cumulative alcohol intake <500 kg (mild to moderate alcohol intake). (8) Patients free of other types of hepatitis, including hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease, and (9) patients who consented to the study.

With regard to the clinical features of 200 patients at the start of IFN monotherapy, there were 103 men and 97 women, aged 27–77 with a median age of 62 years. The median ALT level was 80 IU/L (range, 6–487 IU/L), and the median platelet count was $13.0 \times 10^4/mm^3$ (range, 3.8×10^4–$28.0 \times 10^4/mm^3$). The median viremia level was 1,200 KIU/ml (range, 5–>5,000 KIU/ml) (Table I). Furthermore, 162 of the 200 patients (81%) received IFN-alpha monotherapy by three times per week; the remaining 38 patients (19%) received IFN-alpha monotherapy that included an initial daily administration in the first 8 weeks, followed by three times per week. A median IFN dose per day of 3 million units (MU, range; 3–6 MU) was administered during a median period of 74 weeks (range; 2–118 weeks). Of the 200 patients, 40 had not achieved sustained virological response with prior therapy of IFN plus RBV, and especially 27 patients of them had been treated with adequate combination therapy for at least 24 weeks (median, 43 weeks; range, 24–73 weeks).

Efficient treatment represented normalization of ALT levels (normal reference ranges: 6–50 IU/L) and AFP levels (normal reference ranges: ≤20 μg/L) during and at the end of 48-week treatment protocol.

The study protocol was approved by the Human Ethics Review Committee of Toranomon Hospital.

Laboratory Investigations

Blood samples were obtained at least once every month from the commencement of treatment, and were tested for ALT and AFP levels. The serum samples were frozen at −80°C within 4 hr of collection and then thawed at the time of measurement. HCV genotype was determined by PCR using a mixed primer set derived from the nucleotide sequences of NS5 region [Chayama et al., 1993]. HCV-RNA level was measured quantitatively by PCR (Cobas Amplicor HCV monitor v 2.0 using the 10-fold dilution method, Roche Diagnostics, Indianapolis, IN) at the commencement of treatment. The lower detection limit of the assay was 5 KIU/ml.

Detection of Amino Acid Substitutions in Core Region

With use of HCV-J (accession no. D90208) as a reference [Kato et al., 1990], the sequence of 1–191 aa in the core protein of genotype 1b was determined, and it was compared with the consensus sequence constructed on 50 clinical samples [Akuta et al., 2005a] for detecting substitutions at aa 70 of arginine (wild) or glutamine/histidine (mutant) and aa 91 of leucine (wild) or methionine (mutant). In the present study, aa substitutions of the core region were analyzed by direct sequencing [Akuta et al., 2005a, 2006]. The PCR genotyping could be performed in 193 patients; the remaining seven patients could not be analyzed due to the lack of adequate serum samples obtained before treatment.

Histopathological Examination of the Liver

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim Silverman

TABLE I. Patient Profile and Laboratory Data at Commencement of Interferon Monotherapy in 200 Patients Infected With HCV Genotype 1b

Demographic data	
Number of patients	200
Sex (M/F)	103/97
Age (years)*	62 (27–77)
History of blood transfusion	81 (40.5%)
Family history of liver disease	58 (29.0%)
Body mass index (kg/m^2)*	22.8 (15.6–32.9)
Laboratory data*	
Serum aspartate aminotransferase (IU/L)	69 (18–756)
Serum alanine aminotransferase (IU/L)	80 (6–487)
Serum albumin (g/dl)	3.7 (2.6–4.4)
Gamma-glutamyl transpeptidase (IU/L)	49 (11–368)
Leukocyte count ($/mm^3$)	4,000 (1,700–8,100)
Hemoglobin (g/dl)	13.9 (8.9–17.3)
Platelet count ($\times 10^4/mm^3$)	13.0 (3.8–28.0)
Indocyanine green retention rate at 15 min (%)	20 (4–62)
Serum iron (μg/dl)	146 (37–322)
Serum ferritin (μg/L)	136 (<10–1,308)
Creatinine clearance (ml/min)	99 (13–167)
Level of viremia (KIU/ml)	1,200 (5–>5,000)
Alpha-fetoprotein (μg/L)	9 (2–398)
Total cholesterol (mg/dl)	165 (15–296)
High-density lipoprotein cholesterol (mg/dl)	45 (21–80)
Low-density lipoprotein cholesterol (mg/dl)	96 (43–237)
Triglycerides (mg/dl)	93 (46–228)
Uric acid (mg/dl)	5.4 (2.8–9.4)
Fasting blood sugar (mg/dl)	97 (67–228)
Histological findings	
Stage of fibrosis (F1/F2/F3/F4/ND)	45/42/35/19/59
Hepatocyte steatosis (none to mild/moderate to severe/ND)	90/24/86
Treatment	
Interferon dose (million units/day)	3 (3–6)
Presence of initial daily interferon administration	38 (19.0%)
Amino acid substitutions in the core region[a]	
aa 70 (wild/non-wild/ND)	118/72/3
aa 91 (wild/non-wild/ND)	124/69/0
aa 70 and aa 91 (double wild/non-double wild/ND)	76/115/2

Data are number and percentage of patients, except those denoted by *, which represent the median (range) values.
Two patterns of mutant and competitive are indicated as non-wild. The pattern of wild at aa 70 and wild at aa 91 was evaluated as double wild-type, and the other patterns were non-double wild-type. ND, not determined.
[a]Amino acid substitutions were evaluated in 193 patient using pretreatment sera by direct sequencing.

needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo, Japan), fixed in 10% formalin, and stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. All specimens for examination contained six or more portal areas. Histopathological diagnosis was made by an experienced liver pathologist (HK) who was blinded to the clinical data. Chronic hepatitis was diagnosed based on histopathological assessment according to the scoring system of Desmet et al. [1994].

Follow-Up

Clinical and laboratory assessments were performed at least once every month from the commencement of treatment. Adverse effects were monitored clinically by careful interviews and medical examination at least once every month. Patient compliance with treatment was evaluated with a questionnaire. Blood samples were also obtained at least once every month from the commencement of treatment, and were also analyzed for levels of ALT and AFP at various time points. Follow-up time represented the time from the start of treatment until the stop of treatment, or until the last visit.

Statistical Analysis

Analysis of efficacy of treatment was performed on an intention to treat basis. The χ^2 test, Fisher's exact probability test, and Mann–Whitney's *U*-test were used to compare the background characteristics between groups. The cumulative ALT normalization rates were calculated using the Kaplan–Meier technique; differences between the curves were tested using the log-rank test. Statistical analyses of ALT normalization according to groups were calculated using the period from the commencement of IFN monotherapy. Stepwise Cox regression analysis was used to determine independent predictive factors that were associated with ALT normalization within 48 weeks after the commencement of treatment. The odds ratios and 95% confidence intervals (95%CI) were also calculated. Potential predictive factors associated with ALT normalization