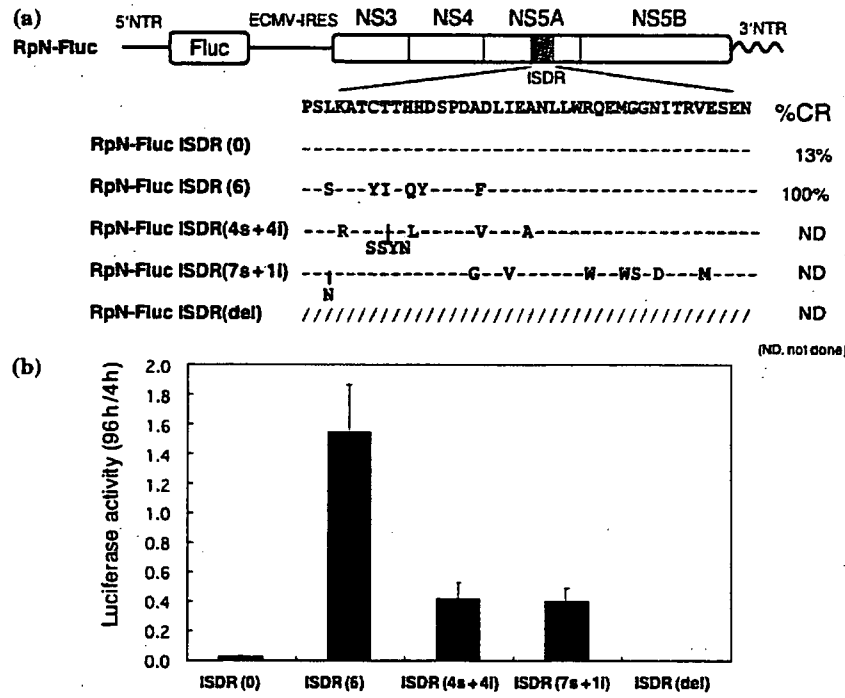

Fig. 3 Various patterns of ISDR sequences with multiple mutations all result in enhancement of HCV replication *in vitro*. (a) Alignment of ISDR sequences in HCV-N replicons with diverse ISDR sequences with multiple mutations observed in patients. In order to demonstrate the effect of deletion of the ISDR, an HCV-N replicon with complete deletion of the ISDR was engineered. (b) Replication capacity of three replicons with multiple ISDR mutations: all were greatly enhanced. In contrast, the replication capacity of replicons with deletion of the entire ISDR was almost completely abrogated. Values correspond to the ratio of the luciferase activity measured 96 and 4 h after transfection. The 4-h value was used to correct for different transfection efficiencies because at this time only luciferase translated from the input RNA was measurable.

introduced into the ISDR of RpN-Fluc (Fig. 4a) [34]. As shown in Fig. 4b, although the replication capacity of most of the replicons was quite low even after the introduction of ISDR mutations, the replication capacity of RpN-Fluc-P2209L was strongly enhanced to almost 20-fold more than that of RpN-Fluc-ISDR(0) (RpN-Fluc-ISDR(0): 387 ± 60, RpN-Fluc-ISDR(1)-P2209L: 6111 ± 500, RpN-Fluc-ISDR(1)-T2216A: 263 ± 20, RpN-Fluc-ISDR(1)-I2227V: 160 ± 50, RpN-Fluc ISDR(1)-H2218R: 703 ± 270, RpN-Fluc-ISDR(1)-H2218C: 241 ± 80, RpN-Fluc-ISDR(1)-A2224V: 580 ± 90).

Finally, using the ISDR-6aa mutant replicon [RpN-Fluc-ISDR(6)] with high replication capacity as the starting material, we determined the specific sites that critically reduced replication by systematically reversing each mutant sequence (Fig. 5a). As demonstrated in Fig. 5b, each back mutation lowered replication capacity to a different extent. Among them, the Q2218H mutation greatly weakened replication capacity (RpN-Fluc-ISDR(0): 101 ± 40, RpN-Fluc-ISDR(6): 11050 ± 690, RpN-Fluc-ISDR(5)-S2211L: 5123 ± 460, RpN-Fluc-ISDR(5)-Y2215C: 3157 ± 480, RpN-Fluc ISDR(5)-I2216T: 5501 ± 1200, RpN-Fluc-ISDR(5)-Q2218H: 404 ± 80, RpN-Fluc-ISDR(5)-Y2219H: 2094 ± 250, RpN-Fluc-ISDR(5)-F2224A: 2795 ± 550).

DISCUSSION

In this study, we demonstrated that the ISDR sequences regulate the replication capacity of HCV replicons, depending on IFN response-related structures. Furthermore, a mapping study revealed that the influence of each ISDR mutation on replication was site-specific, and the critical sites modulating replication *in vitro* coincided with those strongly associated with the IFN response *in vivo*.

As demonstrated in Figs 2 and 3, ISDR sequences regulated replication of HCV replicons, depending on the number of mutations in the region. Even though few mutations (0 to 3 codon changes) in the ISDR did not enhance replication, multiple mutations (resulting in six substitutions, seven substitutions plus one insertion, and four substitutions plus four insertions) all resulted in replication enhancement, excepting artificially engineered total ISDR deletion that abrogated replication capacity. These results were consistent between two replicon clones, HCV-N and HC-J4, indicating that a relationship between the number of ISDR mutations and virus replication *in vitro* may be found widely, beyond specific HCV isolates. Meanwhile, the finding that complete deletion of the ISDR abrogated replication capacity may indicate that maintenance of the conformation of the NS5A

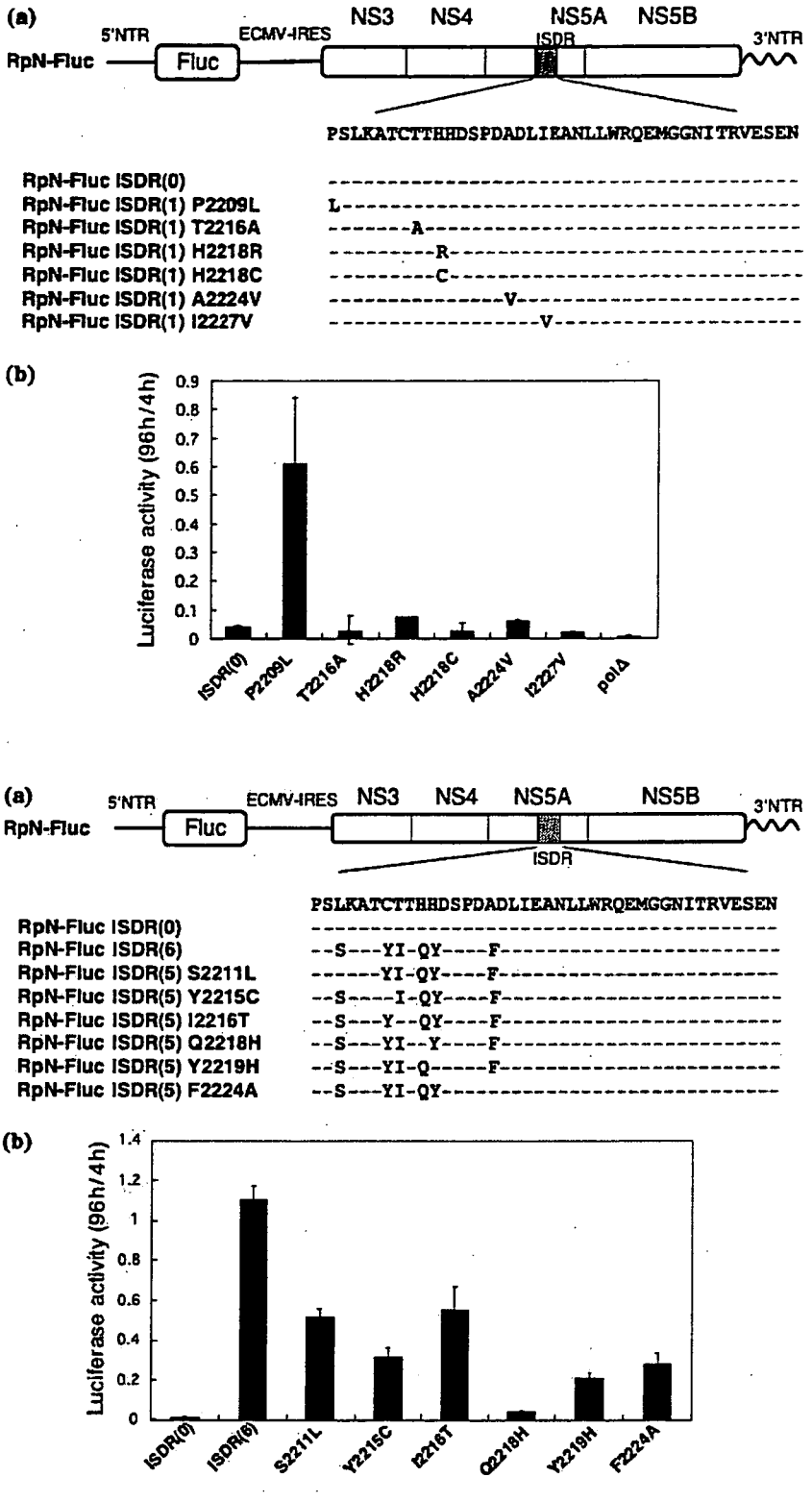

Fig. 4 Determination of mutational points in the ISDR that critically upregulates HCV replication *in vitro*. Alignment of ISDR sequences in the HCV-N replicon with various single amino acid substitutions. These individual mutations were selected for this study because of their frequent appearance in clinical infections. Transient replication capacity of replicons with various single codon changes in the ISDR was analysed by luciferase assay. Although a parental replicon possessing no amino acid mutations in the ISDR had low luciferase activity, the replication capacity increased 20-fold upon introduction of P2209L. In contrast, no other mutation significantly altered replication.

Fig. 5 Determination of mutational points in the ISDR that critically downregulate HCV replication *in vitro*. (a) Alignment of ISDR sequences in the HCV-N replicon with five amino acid substitutions in ISDR. In order to determine the position that critically downregulates replication of a highly replication-competent replicon with six codon changes in the ISDR, we systemically introduced back mutations. (b) Transient replication capacities of replicons with five codon changes in the ISDR were analysed by luciferase assay. Each back mutation lowered replication capacity to a different degree and, in particular, the Q2218H substitution reduced replication capacity greatly.

protein, with inclusion of the ISDR, is required for the replication of the HCV genome. Nevertheless, the need for ISDR for HCV replication should be confirmed in further studies, because a previous study reported that a partial deletion in NS5A, including the ISDR, rather supported replication [26]. This paradox, however, indicates that NS5A conformation has a key role in HCV replication.

At the same time, the effect of mutations in the ISDR on replication was site-specific. The introduction of single codon changes into the wild-type ISDR revealed that substitution of

codon 2209 greatly enhanced replication, almost 20-fold, indicating this site had a great influence on the regulation of replication (Fig. 4). On the other hand, in the experiments using the highly replication-competent replicon with six codon changes in the ISDR as starting material, the influence of reversion of each change on replication also was site-specific, and reversion of the change at codon 2218 decreased replication capacity most effectively. Importantly, codon 2209 was revealed in the clinical analysis to be one of the most important sites determining the IFN response, and codon 2218 was the site most frequently mutated among all codons in the ISDR [10,34]. Collectively, it is considered that the replication of HCV replicons reflects the ISDR sequences related to IFN response and virus replication. From these results, the HCV replicon system reflecting clinical findings could be a powerful tool to analyse the actual NS5A-ISDR function in HCV replication. For further confirmation of the relationship between HCV replication *in vitro* and that *in vivo*, we are now investigating how various NS5A-ISDR-mutant replicons respond to IFN.

Even though the HCV replicon reflects the ISDR sequences related to IFN response and virus replication, why does the influence of ISDR mutations on virus replication *in vitro* and *in vivo* seem to be contrary? In order to answer the question, the mechanism(s) by which adaptive mutations enhance replication must be clarified. Whether adaptive mutations scattered throughout the viral genome all have the same function is as yet unknown, but the functional role of the NS5A-serine cluster region recently has been investigated intensively, and mutations in the region were demonstrated to modify the phosphorylated state of NS5A proteins [26,29–31,37]. NS5A is a phosphoprotein, and has two phosphorylated isoforms: the basal phosphorylated form (p56) and the hyperphosphorylated form (p58). The NS5A protein is part of the HCV replication complex, and p56 must bind directly to a SNARE-like protein, hVAP-A, expressed in the ER membrane, in order to form the HCV replication complex [29,30,38]. On the other hand, p58 dissociates the NS5A protein from hVAP-A, preventing the HCV replication complex from forming on the ER membrane, and has been implicated in playing a certain role in virus particle assembly or excretion of virus from the cells. Thus, adaptive mutations have been proposed to prevent disassociation of the replication complex, freezing the viral life cycle at the RNA replication stage and inhibiting virion production and viral spread [39]. Although it is not clear whether the ISDR has the same function as the serine cluster region, it is possible that the ISDR has some influence on this function, because it is located adjacent to the serine cluster region and ISDR mutations enhance HCV replication synergistically with serine cluster mutations. If this is the mechanism, the inverse relationship of the *in vitro* and *in vivo* effects can be explained, because the HCV-RNA titre might reflect virion production and their secretion into the serum, while replicon replication reflects intracellular viral replication. However, further studies are needed to test this hypothesis.

On the other hand, there is a big difference in the environment of cultured cells and the human liver. The human liver contains many immune cells, such as cytotoxic T lymphocytes, natural killer cells and dendritic cells, and a variety of cytokines, including endogenous IFN-alpha/beta produced by these cells [40]. Consequently, immunological signal pathways such as the JAK-STAT pathway and downstream interferon-inducible genes are thought to be always active to some extent in the human liver [11], in contrast to cultured cells that are not affected by immune cells or cytokines. In the innate immune system, there are two major pathways: the primary anti-viral pathway not requiring type I IFN, and the second anti-viral pathway requiring IFN for its activation [41]. It is considered that the JAK-STAT pathway is involved in the second pathway, while IRFs are involved in the primary pathway. Because of the differences in these environments, it has been suggested that the primary pathway should have a dominant role as an innate immunity in cultured cells, in contrast to the human liver where the second pathway might have an important role. We have shown recently that expression of IRF-1 was greatly suppressed in replicon-harbouring cells, or even in replicon-cured cells, from which replicons had been eliminated by exogenous IFN treatment, compared with naïve Huh-7 cells [42]. When IRF-1 was induced by plasmid expression, replicon replication was greatly attenuated, indicating that downregulation of the IRF-1 pathway was necessary and was a key pathway regulating replicon replication. On the other hand, we also have shown in the previous study that the need for NS5A-adaptive mutations for replication decreases when IRF-1-suppressed cured cells are used [33], indicating that NS5A-adaptive mutations and IRF-1 suppression can effectively complement one another to permit replication. Thus, the IRF-1 pathway is also considered as a candidate target of NS5A adaptive mutations, including the serine cluster region and ISDR.

In conclusion, we have demonstrated that the numbers of mutations in the ISDR regulate the replication of replicons, suggesting that ISDR sequences associated with IFN sensitivity influence the replication of replicons *in vitro*. Moreover, specific sites in the ISDR critically regulate replication, showing that the effects of mutations in the ISDR vary according to the site. Although the numbers of analysed ISDR sequences were still small, our results indicate that the replicon system might be used as a tool to clarify the molecular mechanism of NS5A-ISDR to modulate HCV replication.

CONFLICT OF INTEREST

We do not have a commercial or other association that might pose a conflict of interest.

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Divergent activities of interferon-alpha subtypes against intracellular hepatitis C virus replication

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Abstract

Backgrounds: Interferon (IFN)-alpha is represented by several structurally related subtypes that show different antiviral and anti-tumor effects. Here, we analyzed differential effects of IFN-alpha subtypes on intracellular hepatitis C virus (HCV) replication using HCV subgenomic replicon system as a model.
Methods: Huh7 and HeLa cells supporting expression of HCV replicon were treated with various concentrations of five recombinant human IFN-alpha subtypes 1, 2, 5, 8, and 10, and with IFN-alpha con1. The effects of IFNs on various cell-signaling pathways were assayed by using ISRE-, GAS-, AP1-, NF-kappa B-, CRE-, and SRE-luciferase reporter plasmids.
Results: Each IFN-alpha subtype suppressed HCV replication in a dose-dependent manner. Among them, IFN-alpha8 was the most effective, while IFN-alpha1 was the least effective with 50% inhibitory concentrations of 0.123 IU/ml versus 0.375 IU/ml, respectively. These differential effects against HCV replication did not correlate with levels of the IFN-responsive ISRE or GAS reporter activities, nor they did activate the other reporters, AP1, NF-kappa B, CRE and SRE.
Conclusion: There were divergent effects of IFN-alpha subtypes against HCV replication that may be through JAK-STAT-independent pathways. Exploring further mechanisms of action may elucidate IFN-mediated cellular antiviral mechanisms.

Keywords: Interferon subtype; Consensus interferon; Hepatitis C virus replicon; ISRE; GAS; Drug synergy

1. Introduction

Hepatitis C virus (HCV) is a world-wide health care problem causing a spectrum of liver disease raging from asymptomatic carrier state to liver cirrhosis and hepatocellular carcinomas [1]. Currently available anti-HCV treatments are based on administration of a major antiviral cytokine, type I interferons (IFN-alpha or -beta). Among them, therapies with recombinant interferon-alpha subtype 2a or 2b and their chemically modified derivatives, PEG-IFNs, are the de facto standard in the current clinical therapeutics. Their effectiveness, however, remains around 50% of patients treated [2].

IFN plays a central role in eliminating viruses not only as therapeutic applications but also as natural cellular antiviral defense mechanisms [3,4]. Also in HCV, a DNA microarray analysis of chimpanzee liver experimentally inoculated with

Abbreviations: AP1, activator protein 1; CRE, cAMP response element; GAS, interferon-gamma activation sequence; HCV, hepatitis C virus; IFN, interferon; ISRE, interferon stimulation response element; JAK, janus kinase; SRE, serum response element; STAT, signal transducers and activators of transcription

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HCV revealed that expression of the interferon-stimulated genes (ISGs) was the principal reaction during the course of the viral infection and its clearance [5].

Type I IFNs are represented by the leukocyte and fibroblast types [6]. IFN-beta is encoded by a single gene, whereas IFN-alpha is represented by a large family of structurally related genes expressing 13 subtypes [7–10]. Among 13 proteins produced from these genes, IFN-alpha13 is identical to that produced from IFN-alpha1 gene. Thus, there are 12 different human IFN-alpha proteins. Several studies have shown that IFN-alpha subtypes have different properties in their mode of expression and the effects on the target cells depending on the cell types and on the IFN inducer used. In response to virus infection to murine cells, expression of IFN-alpha1, -alpha4, and -alpha6 mRNA are induced differently between cell types [11]. An analysis of mRNA expression in liver of chronic hepatitis C patients has shown that the expression patterns of each IFN subtype are different from those of normal controls [12].

In addition to the difference of the expression profile, IFN-alpha subtypes show different biological activities on their target cells. Each IFN-alpha subtype shows distinct patterns of antiviral, growth inhibitory, and other biologic activities [13–16]. Regarding antiviral activity, studies using murine encephalomyelitis virus and various human cell lines have shown that IFN-alpha8 was the most potent, while IFN-alpha1 was the least potent [17]. These reports have made us speculate that there might be subtype-specific IFN-signaling pathways.

Actions of type I IFN are initiated by binding of the IFN-alpha receptors (IFNARs) expressed on cell surface. The binding of IFN to their receptor activates two kinases; janus kinase 1 (JAK1) and the tyrosine kinase 2 (TyK2), which associate with the intracellular domain of IFNARs). These receptor-kinases activate the SH2 domain of the signal transducer and activator transcription factors (STATs). The phosphorylated STAT1 and STAT2 recruit IFN regulatory factor (IRF)-9 to form the IFN-stimulated gene factor-3 (ISGF-3) and activates expression of the ISGs by binding the interferon stimulation response element (ISRE) in their promoter/enhancer domain [18,19].

Studies on HCV replication have been hampered by the lack of efficient cell culture models. The problem was partly overcome by the development of an HCV subgenomic replicon system, which simulates cellular autonomous replication of HCV genomic RNA [20]. More recently, an efficient HCV cell culture system has been reported [21]. Replication of HCV replicon can be abolished by treatment with type I and type II IFNs [22–24]. These findings indicate that the IFN receptor-mediated cellular responses are intact in the HCV replicon system. In the present study, we have analyzed effects of five IFN-alpha subtypes on intracellular HCV replication using HCV subgenomic replicon system, and investigated on the difference of mechanisms of action.

2. Materials and methods

2.1. Cell culture

Huh7 and HeLa cells were maintained in Dulbecco's modified minimal essential medium (Sigma, St. Louis, MO) supplemented with 2 mM L-glutamine and 10% fetal calf serum at 37 C under 5% CO_2. Huh7 cells containing HCV replicon were cultured in medium containing 500 g/ml G418 (Nakalai, Kyoto, Japan).

2.2. HCV replicon constructs and transfected cell lines

An HCV subgenomic replicon plasmid, pHCVIbneo-delS was derived from an infectious HCV clone, HCV-N, genotype 1b [24]. The replicon was reconstructed by substituting the neomycin phosphotransferase gene with a fusion gene comprising the firefly luciferase and neomycin phosphotransferase (pRep-Feo) [25,26]. Another replicon construct, pSGR-JFH1 was derived from an infectious HCV clone, JFH-1, genotype 2a [27]. The replicon was reconstructed by substituting neomycin phosphotransferase gene with a fusion gene comprising of renilla luciferase and neomycin phosphotransferase (pRep-Reo). RNA was synthesized from linearized replicon plasmids using T7-polymerase (Promega, Madison, WI) and transfected into HeLa cells. After culture in the presence of G418, cell lines stably expressing the replicon were established (Huh7/Rep-Feo and HeLa/Rep-Reo). The expression levels of the replicon in the cell lines were determined by measuring luciferase activities of the cell lysates.

2.3. Reporter plasmids

A plasmid, pISRE-TA-Luc (BD Biosciences Clontech, Palo Alto, CA), expressed the Fluc gene under the control of the ISRE [28]. pGAS-TA-Luc, pAP1-TA-Luc, NF-kappa B-TA-Luc pSRE-TAL-luc, and pCRE-TAL-luc (BD Biosciences Clontech) are also used to see the expressed Fluc gene under GAS, AP1, NF-kappa B, SRE and CRE. A plasmid, pRL-CMV (Promega), which expresses the renilla luciferase gene under the control of the cytomegalovirus early promoter/enhancer, was used to normalize transfection efficiency.

2.4. Recombinant IFN-alpha subtypes

Recombinant human IFN-alpha1, -alpha2, -alpha5, -alpha8, and -alpha10 were prepared via the pET-3a-BL21 system (Novagen, Madison, WI) as described previously [29]. IFN-alpha activities were measured in a cytopathic effect (CPE) reduction assay using Sindbis virus-infected FL cells, and the IFN titers were standardizes in international units (IU/ml) by comparison with the WHO standard [29]. Cells were seeded in 96-well plates and incubated with two-fold dilutions of each IFN-alpha subtype. After incubation for 72 h, The CPE in each well was determined, and the specific

antiviral activity was expressed in terms of the mean 50% inhibitory concentration (IC50) of each IFN-alpha subtype on a weight basis. The activities were 3.0×10^6 IU/mg for subtype alpha1, 7.2×10^7 IU/mg for alpha2, 4.0×10^7 IU/mg for alpha5, 2.9×10^8 IU/mg for alpha8, and 4.9×10^7 IU/mg for alpha10. As regards IFN-alpha2, because of glycosylation of the naturally occurring subtype that may influence its biologic activity, we also purified natural IFN-alpha2 from cultures of BALL-1 cells stimulated with Sendai virus instead of producing a recombinant product. Recombinant interferon-alpha con1 was kindly provided by Yamanouchi Co. Ltd. (Tokyo, Japan). Specific activity of IFN-alpha con1 was 2.05×10^9 IU/mg [30].

2.5. Luciferase assays

Luciferase assays were done as described previously [28,31]. Typically, the replicon-expressing cells were seeded on a 24-well plate at a density of 1×10^4 cells per well. IFNs were applied to the culture medium at various concentrations (0.01–100 IU/ml). After 48 h of culture, expression levels of HCV replicon were measured by luciferase assay using the Bright-Glo Luciferase Assay System (Promega) or the Renilla Luciferase Assay System (Promega) and a luminometer, TD-20/20 (Turner Designs, Sunnyvale, CA). Assays were done in triplicate and the results were expressed as means ± S.D. To perform dual reporter assays, cells were seeded onto 24-well plate at 5×10^4 cells per well, and transfected with 400 ng of pISRE-TA-Luc and pRL-CMV (1 ng for Huh7, 10 ng for HeLa, respectively) using 1.2 l of FuGene6 (Roche, Indianapolis, IN) per well. IFN-alpha subtypes were applied at 24 h after the transfection. Cells were harvested at 24 h after the addition of IFNs. The dual reporter assays were done using the Dual-Luciferase Reporter Assay System (Promega).

2.6. Western blot analysis

Ten micrograms of nuclear extract lysate was separated in a NuPAGE 10% Bis–Tris Gel (Invitrogen) and blotted onto the PVDF Western Blotting Membrane (Roche). The membranes were incubated with mouse monoclonal anti-NS5A antibody followed by POD-labeled anti-mouse IgG antibody. Chemiluminescence was detected using the ECL Western blotting Analysis System (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's protocol.

2.7. Analyses of drug synergism

The effects of treatment of Huh7/Rep-Feo cells with the IFN subtypes, alone and in combination, were analyzed according to classical isobologram analysis [32,33]. Dose–inhibition curves of IFN subtypes A and B were drawn, with the two drugs used alone or in combination. In each drug combination, the concentrations of IFN subtypes A and B that inhibited HCV replication to 50% (IC50) were plotted against the fractional concentration of each IFN subtype on the *X*- and *Y*-axes, respectively. The combination index, CI, for each combination was calculated using the following formula:

$$CI = \frac{IC50(\text{IFN subtypes A and B combined})}{IC50\ (\text{IFN subtype A alone})} + \frac{IC50(\text{IFN subtypes A and B combined})}{IC50\ (\text{IFN subtype B alone})}$$

For such plots, the combined effects of two drugs can be assessed as an either additive effect, indicated by CI = 1, synergy, indicated by CI < 1, or antagonism, indicated as CI > 1.

2.8. Reverse transcription-polymerase chain reaction (RT-PCR) assay

Total cellular RNA was extracted from cells using ISOGEN (Wako, Osaka, Japan). Two micrograms of total cellular RNA was used to synthesize cDNA from each sample using the SuperScript II (Invitrogen) reverse transcriptase. PCR was done in a mixture containing 1 1 cDNA sample; 1.25 mM $MgCl_2$; 0.5 M of each primer; 0.4 mM of dNTP and BD advantage2 PCR Kit (BD Biosciences Clontech, Palo Alto, CA). Primers used were as follows: IFNAR-1, sense, 5 -AGT GTT ATG TGG GCT TTG GAT GGT TTA AGC-3 , IFNAR-1, antisense, 5 -TCT GGC TTT CAC ACA ATA TAC AGT CAG TGG-3 , IFNAR-2, sense, 5 -ATT TCC ATC TAT TGT TGA GG-3 , IFNAR-2, antisense, 5 -CAC TTT CTT CTT TCT GTT GA-3 , beta-actin, sense, 5 -CAC CAT GGA TGA TGA TAT CGC CGC GCT CGT-3 , and beta-actin, antisense, 5 -GAA GCA TTT GCG GTG GAC GAT GGA GGG GCC-3 . The PCR products were separated on 1% agarose gel, stained with etidium bromide, and visualized by UV transilluminator.

2.9. Statistical analyses

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

3. Results

3.1. Analysis of suppressive effects of the IFN-alpha subtypes on expression of HCV replicon in Huh7 cells

Huh7/Rep-Feo cells were treated with various concentrations of IFN subtypes (0.01–100 IU/ml). After 48 h of the treatment, the levels of HCV replication were analyzed by luciferase assay. Replication level of HCV replicon was significantly suppressed by each IFN subtype in dose-dependent manner (Fig. 1A). Fifty percent inhibitory concentrations (IC50) of the IFN subtypes were calculated as 0.375 IU/ml (125 pg/ml) for alpha1, 0.220 IU/ml (3.05 pg/ml) for alpha2, 0.238 IU/ml (5.94 pg/ml) for alpha5, 0.124 IU/ml

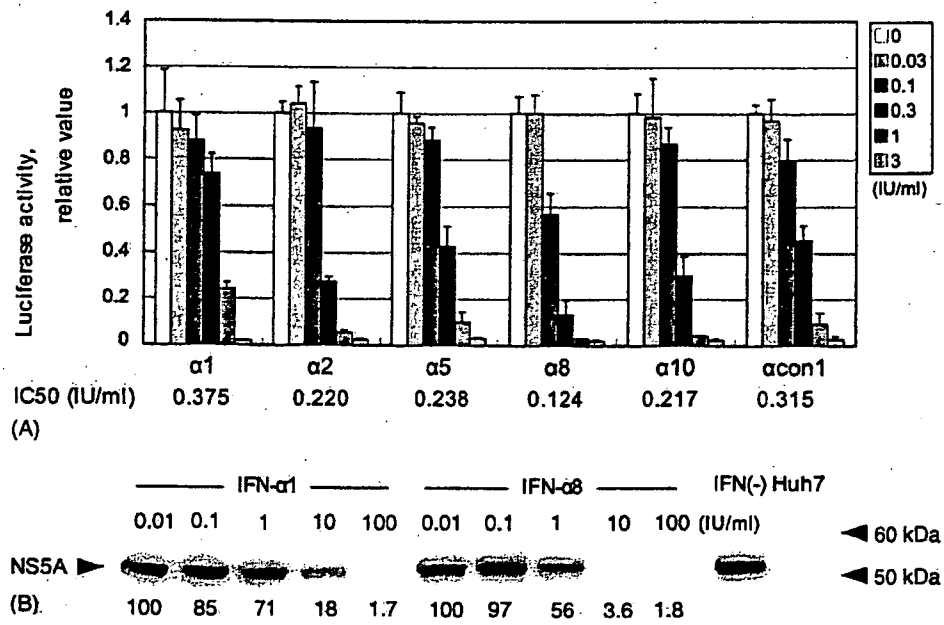

Fig. 1. Analysis of antiviral effect of the IFN-alpha subtypes using HCV replicon Huh7 cells: (A) luciferase activities of Huh/Rep-Feo were cultured in the presence of indicated concentration of IFN subtypes. Luciferase activities were measured at 48 h after culture. Values indicate relative luciferase activities as percents of IFN-negative controls. Error bars indicate mean ± S.D. (B) Western blotting. Huh7/Rep-Feo cells were treated with indicated concentrations (numbers on the top) of IFN-alpha1 and -alpha8. Total cell lysate was harvested after 48 h, and Western blotting was done using primary antibody directed against NS5A. Numbers on the bottom indicate densitometric values displayed as percents of an IFN-negative control.

(0.429 pg/ml) for alpha8, 0.217 IU/ml (4.43 pg/ml) for alpha10, and 0.315 IU/ml (0.153 pg/ml) for alpha con1, respectively. As indicated, expression of HCV replicon in Huh7 cells was the most sensitive to IFN-alpha8, and the least to IFN-alpha1 by titers standardized in IU/ml and in pg/ml. IFN-alpha8 was 1.8 times more sensitive than IFN-alpha2 to suppress HCV replication. Treatment of Huh7/Rep-Feo with a solvent of IFN proteins showed no effects on expression levels of HCV replicon. To confirm the result of IFN-alpha1 and -alpha8, we next carried out Western blotting. Cells were treated with IFN-alpha1 and -alpha8, and total cell lysate was harvested after 48 h. The expression of replicon-derived NS5A protein was dose-dependently suppressed by treatment with IFN-alpha1 and -alpha8, and the velocities of suppression were comparable to those of luciferase activities (Fig. 1B).

3.2. *Effects of IFN subtypes on cellular signal transduction pathways*

We next investigated effects of IFN subtypes on the ISRE and GAS promoter activities by luciferase reporter assay. Plasmids, pISRE-luc and pGAS-luc were transfected into Huh7 cells. Twenty-four hours after transfection, various concentrations of IFN subtypes were added into the medium. Luciferase assay was done at 6 h after addition of IFNs. The ISRE- and GAS-mediated luciferase activities were elevated by dose-dependent manner of each IFNs. However, there was no significant difference in their induction velocities between each IFN subtypes (Fig. 2A). Furthermore, a discrepancy was observed between the suppressive effects of HCV replication and the ISRE and GAS activities; IFN-alpha1 and -alpha8 apparently showed similar activations of ISRE and GAS promoter, while IFN-alpha8 was more effective to inhibit the HCV replication than IFN-alpha1. Therefore, we investigate alternate pathway that may be activated by IFN. Reporter assays were performed by using plasmids, pAP1-luc, pNF-kappa B-luc, pCRE-luc, and pSRE-luc. Luciferase assays after addition of each IFN subtypes showed that neither of AP1, NF-kappa B, CRE, and SRE reporters were activated by IFN treatment (Fig. 2B).

3.3. *Analyses of synergism between IFN subtypes*

It has been reported that interactions between IFN subtypes may influence cellular response to IFN [16]. Our previous study showed that IFN-alpha2 and -alpha8 had synergistic antiviral effect against vesicular stomatitis virus (VSV) infection to HepG2 cells. [29]. To investigate if the IFN subtypes synergistic effects on HCV replication, a classical isobologram analysis was performed [32,33]. Huh7/Rep-Feo cells were treated with IFN-alpha2 or -alpha8 alone, or with IFN-alpha2 and -alpha8 that were mixed with equivalent IC50 ratio, and the dose–effect plots were drown (Fig. 3A). Each concentration of IFN-alpha2 and -alpha8 at 50% inhibition were plotted on the *X*- and *Y*-axes, respectively, to generate an isobologram (Fig. 3B). A plot showing the IC50 of IFN-alpha2 and -alpha8 at 1:1 mixture was located on the line connecting IC50 of IFN-alpha2 or -alpha8 alone, indicating that the effects of the drug

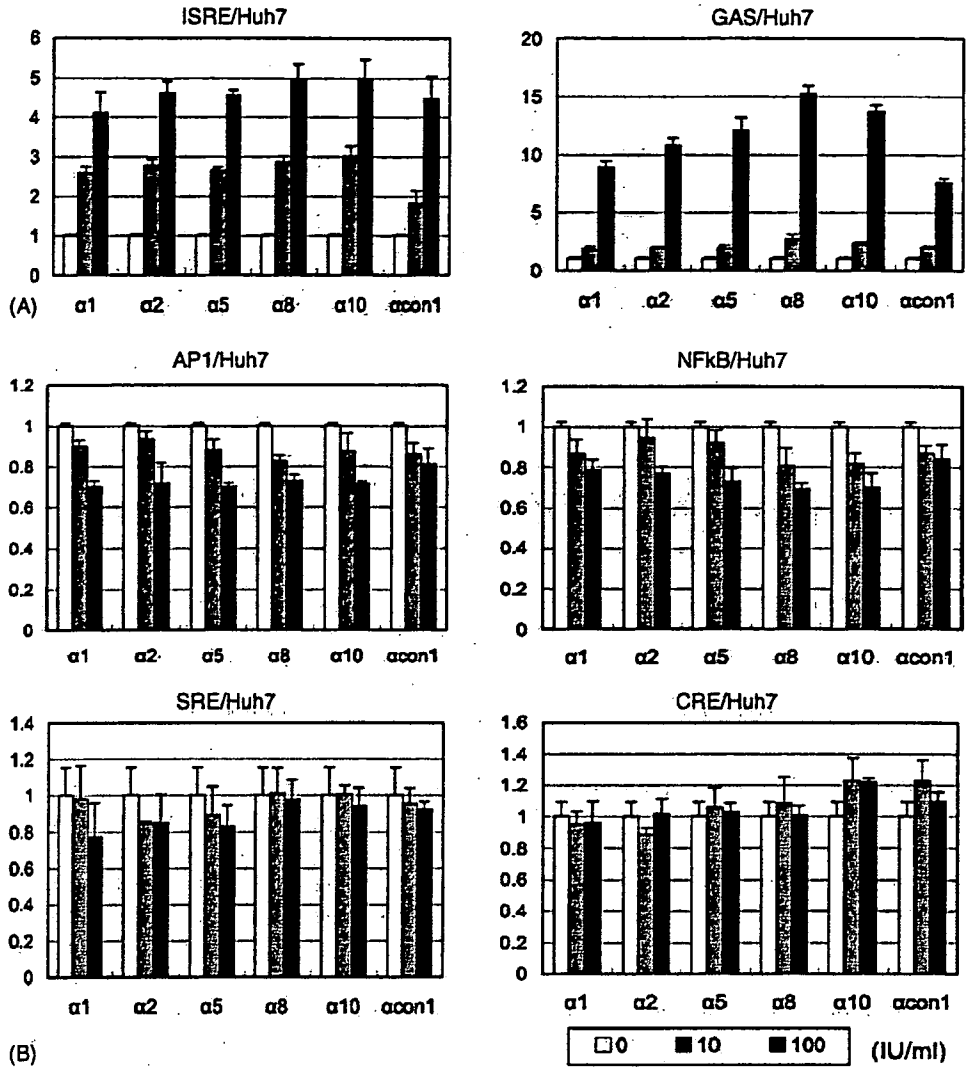

Fig. 2. ISRE, GAS, AP1, NF-kappa B, CRE and SRE reporter assay of Huh7 cells. ISRE-, GAS-, AP1-, NF-kappa B-, CRE-, or SRE-Luc reporter plasmids was transfected into Huh7 with pRL-CMV as a control. Twenty-four hours after transfection, the medium was supplemented with the IFN-alpha subtypes indicated. Dual luciferase assays were done at 6 h after the addition of IFNs. Bars indicate relative reporter activities as percents of IFN-negative controls. Error bars indicate mean ± S.D.

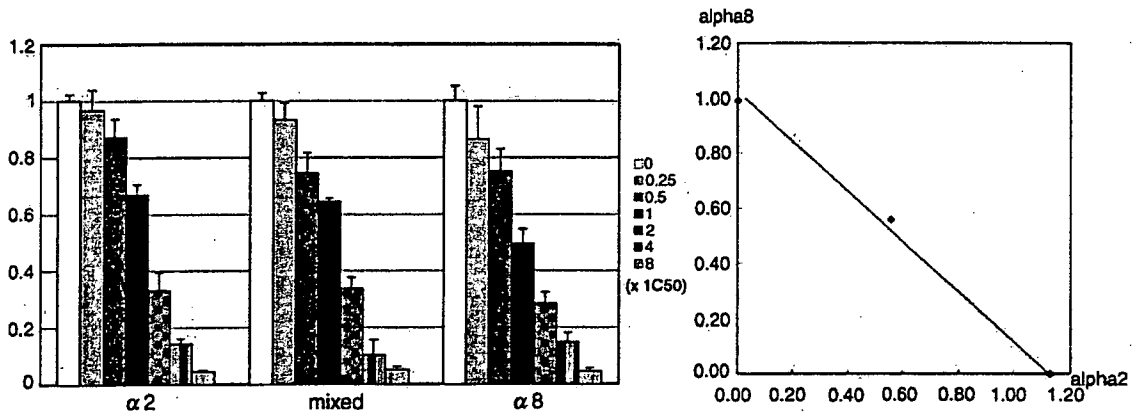

Fig. 3. Analyses of synergism between IFN subtypes 2 and 8: (A) IFNs; IFN-alpha2, -alpha8, mixed, dose modified by IC50 are added to Huh/Rep-Feo. Luciferase activities of Huh/Rep-Feo were measured 48 h after the addition of IFNs. (B) Graphical representation of the isobologram analysis. IC50s were calculated from the values in panel A. Each fractional IC50 for IFN-alpha2 and -alpha8 was plotted on *X*- and *Y*-axes, respectively. The FIC plot for the IFN-alpha2 and -alpha8 combinations of 1:1 falls on a theoretical line of additivity that is drawn between the IS50 plot for each IFN alone, indicating the effects of combination of IFN-alpha2 and -alpha8 is additive.

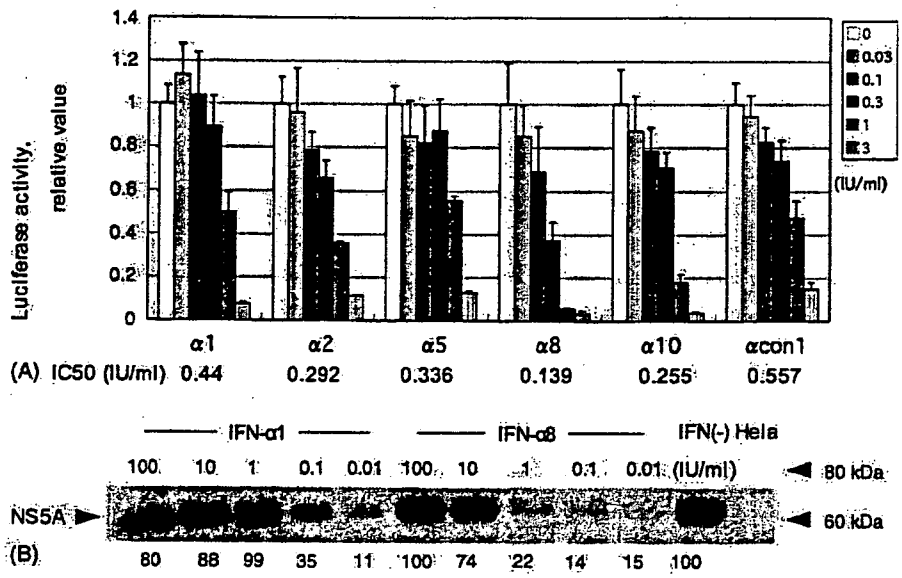

Fig. 4. Analysis of antiviral effect of the IFN-alpha subtypes using HCV replicon HeLa cells: (A) Luciferase activities of HeLa/Rep-Reo were cultured in the presence of indicated concentration of IFN subtypes. Luciferase activities were measured at 48 h after culture. Values indicate relative luciferase activities as percents of IFN-negative controls. Error bars indicate mean ± S.D. (B) Western blotting. HeLa/Rep-Reo cells were treated with indicated concentrations (numbers on the top) of IFN-alpha1 and -alpha8. Total cell lysate was harvested after 48 h, and Western blotting was done using primary antibody directed against NS5A. Numbers on the bottom indicate densitometric values displayed as percents of an IFN-negative control.

combination on intracellular HCV-RNA replication is additive.

3.4. Analysis of antiviral effect of the IFN-alpha subtypes using HCV replicon HeLa cells

Different type of cells may respond to IFN in a different manner possibly depending on the expression profiles of interferon receptors or cellular factors that mediate IFN signal transduction. To investigate the effects of IFN subtypes in a non-hepatocyte cell line, we used HeLa cells expressing chimeric luciferase reporter HCV replicon, HeLa/Rep-Reo. Treatment of HeLa/Rep-Reo cells with IFN subtypes suppressed expression of HCV replicon in dose-dependent manner (Fig. 4A). The IC50 for each IFN subtypes were 0.44 IU/ml (147 pg/ml) for alpha1, 0.292 IU/ml (4.06 pg/ml) for alpha2, 0.336 IU/ml (8.4 pg/ml) for alpha5, 0.139 IU/ml (0.479 pg/ml) for alpha8, 0.255 IU/ml (5.20 pg/ml) for

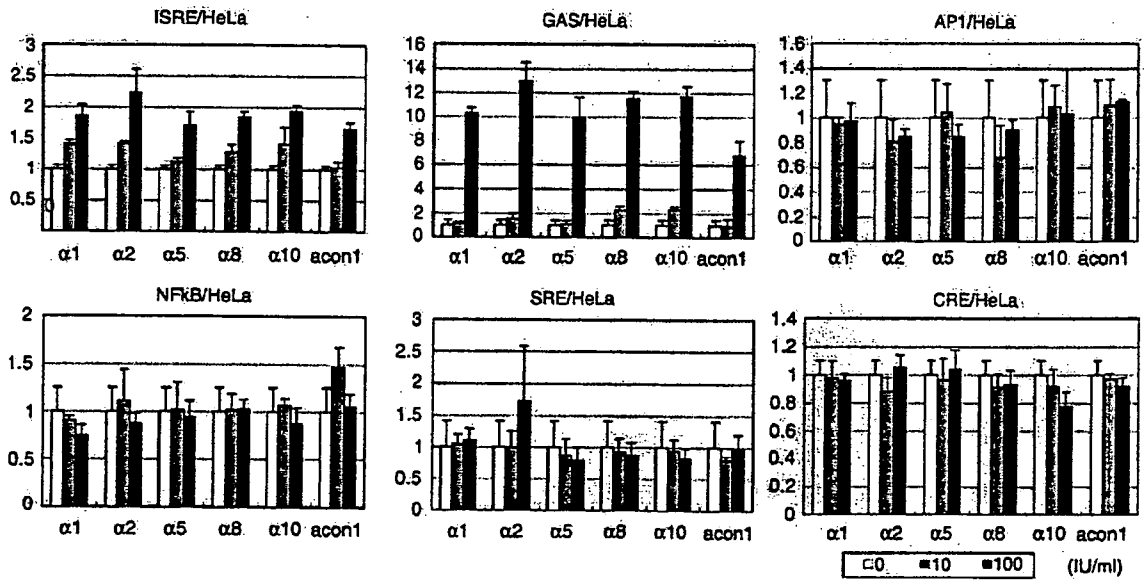

Fig. 5. Reporter assay of HeLa cells. ISRE-, GAS-, AP1-, NF-kappa B-, CRE-, and SRE-Luc reporter plasmids were, respectively, transfected into HeLa cells with pRL-CMV as a control. Twenty-four hours after transfection, IFN subtypes were added onto the medium. Dual luciferase assays were done at 6 h after the addition of IFNs. Bars indicate relative reporter activities as percents of IFN-negative controls. Error bars indicate mean ± S.D.

alpha10, and 0.557 IU/ml (0.271 pg/ml) for alpha con1, respectively. Similarly to the Huh7 cells, IFN-alpha8 was the strongest to suppress expression of HCV replicon. On the other hand, IFN-alpha5 and alpha con1 showed weaker antiviral effects on HCV replicon in HeLa cells than in Huh7 cells. Western blotting of HeLa/Rep-Reo cells treated with IFNs-alpha1 and alpha8 showed dose-dependent suppression of HCV replication, and differential activities of IFN subtypes, which were comparable to that of luciferase activities (Fig. 4B).

Reporter assays were performed by transfecting plasmids expressing ISRE-, GAS-, AP1-, NF-kappa B-, CRE-, and SRE-luciferase reporters into HeLa cells. The ISRE- and GAS-luciferase constructs responded to treatment with IFN subtypes similarly to that in Huh7 cells (Fig. 5). There was no significant difference in induction velocity of ISRE and GAS reporter activities between different IFN subtypes as were seen in Huh7 cells. IFN treatment showed no significant effects on AP1, NF-kappa B, CRE, and SRE activities on HeLa cells.

3.5. Analyses of IFN receptors expression

It is possible that the differences in expression levels of the cell-surface IFN receptors may associate with the response to IFN. We then analyzed expression of the respective subunits of type I IFN receptor mRNAs of Huh7 and HeLa cells by RT-PCR. Type I IFN receptor, IFNAR, is constituted by two subunits; 110 kilo-dalton (kDa) alpha subunit (IFNAR-1), and a 102 kDa beta subunit (IFNAR-2). IFNAR-2 has three isoforms that are translated from alternatively spliced mRNA transcripts; a 40 kDa soluble form of IFNAR-2a, a 55 kDa short form of IFNAR-2b and a 102 KDa long form of IFNAR-2c [34–36]; IFNAR-2c is the authentic beta subunit that is functionally active and coexpressed with IFNAR-1 (Fig. 6A). An RT-PCR analysis of IFN receptors showed that both cell lines expressed IFNAR-1 and IFNAR-2 (Fig. 6B). Although the relative expression levels of IFNAR-2a was slightly higher in Huh7 cells that in HeLa cells, There were no apparent differences in the expression level of the major subunits, IFNAR-1 and IFNAR-2c between Huh7 and HeLa cells.

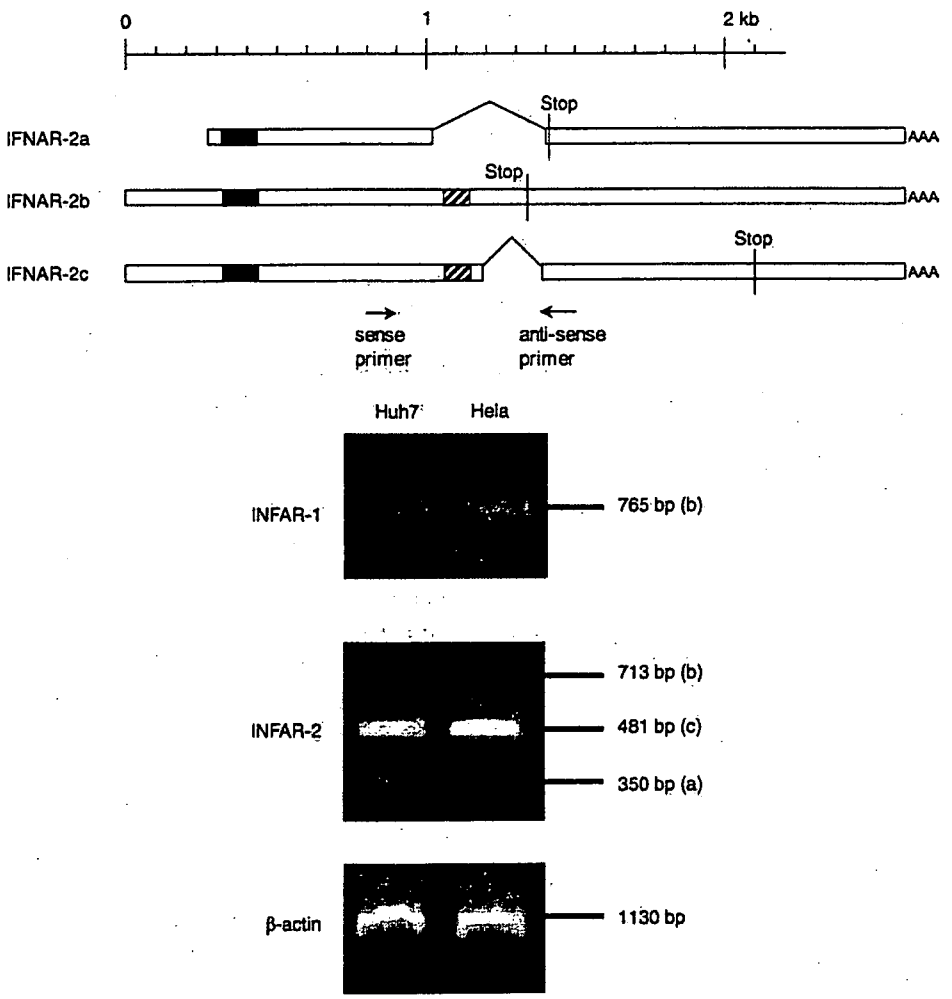

Fig. 6. IFN receptor expression: (A) structures of 3 IFNAR2 mRNA isoforms. Closed boxes indicate the leader peptide domains. The striped boxes indicate the transmembrane domain. Stop codons are indicated by vertical bars. Position of the sense and antisense primers are shown at the bottom. (B). Expression of IFN-alpha receptors in Huh7 and HeLa cells were evaluated by RT-PCR. The top panel: IFNAR-1 PCR-amplified DNA of 765 bp. The middle panel: IFNAR-2 PCR products, (a) 350 bp as IFNAR-2a, (b) 713 bp as IFNAR-2b, and (c) 481 bp as IFNAR-2c in size, respectively. The bottom panel: beta actin DNA.

4. Discussion

In this study, we used two cell lines that support expression of HCV replicon, in which the level of the viral genomic replication can by readily monitored by luciferase reporter assay. We showed that the five IFN-alpha subtypes have different activities to suppress expression of HCV replicon (Figs. 1 and 3). Using two IFN titers standardized in IU/ml and in pg/ml, IFN-alpha8 had the strongest antiviral effect on replicon, while IFN-alpha1 had the weakest effect in both titers. These finding are consistent with those reported by Foster et al. that IFN-alpha8 had the greatest antiviral activity in cells of three human tumor cell lines challenged with murine encephalomyelitis virus [17]. On the other hand, the reporter assay showed that activation of ISRE-dependent promoter, which is the primary signal transduction pathway, showed very similar results between Huh7 and HeLa cells, while the ISRE activities in neither of the cell lines correlated with the anti-HCV activities of the IFN subtypes (Figs. 2 and 4). GAS reporter activity, which bound by phosphorylated STAT1 homodimer, showed similar activation between each IFN subtypes. Other reporter assays, NF-kappa B, CRE, and SRE, showed no activation by the IFNs. These findings suggest that the divergent action of IFN subtypes may be independent of the classical JAK-STAT pathway.

Beside the classical JAK1-STAT1 and -2 pathway, type I IFN activates alternative signaling pathways. JAK2 mediates activity of IFNs as well as JAK1. As for STAT family, dimmers of STAT1:1, STAT3:3, STAT1:3, STAT5:5, and a heterodimer CrkL:STAT5 have been reported to be formed during the IFN-alpha signaling [18,19]. Furthermore, IFN-alpha treatment of cells activates expression of various genes that modulate virus infection and replication in JAK-STAT-independent manner; those include the insulin receptor substrate family, CrkL adaptor, protooncogene Vav, PKC-delta, p38 kinase, ERK 1/2, and PI-3 kinase, although the targets for these signaling pathways have not been well understood (reviewed in [18,19]).

Actions of IFN-alpha is initiated by binding the type I IFN receptors. It has been suggested that biologic activities of different IFN-alpha subtypes correlate with their respective binding affinities to the cells used [37]. Although we have not tested the cell-binding affinity of the IFN subtypes onto their receptor, activation of ISRE promoter, which is triggered by the receptor binding of IFN, did not correlate with their antiviral activities. Furthermore, analyses of IFN receptors by RT-PCR did not find differences in expression profiles the type I IFN receptor subunits, IFNAR1 and three isoforms of IFNAR2 [34–36] in Huh7 and HeLa cells that support expression of HCV replicon (Fig. 6B), suggesting that the differential effects of IFN subtypes may not be due to different expression profiles of their receptor subunits. Alternatively, binding of IFNs onto their receptor might recruit unidentified subunits or adaptor molecules that may activate aberrant signal transduction pathways.

Most studies on actions of the IFN subtypes focused only on the effects of the individual subtypes, and very little is known about their effects in combination [38–40]. On the other hand, because of the existence of multiple IFN subtypes, mutual interactions between the subtypes may be involved in the cellular responses, although these interaction between IFN subtypes are not well understood. Greiner et al. had reported IFN-alpha1 competes with IFN-alpha2 for binding to its receptor [16]. Our previous study also demonstrated additive and antagonistic effect of IFN-alpha subtypes, for instance, IFN-alpha2 and alpha8 had synergistic antiviral effect against VSV virus in HepG2 cells. [29] In our present study, we could not find such synergistic effects of IFN-alpha2 and alpha8 subtypes on cellular HCV replication (Fig. 3B). The result suggests that effects of IFN subtypes and their combination may show different effects depending on the target pathogens.

IFN-alpha con1 is a recombinant IFN that has consensus amino acid sequence of multiple IFN-alpha subtypes on its receptor-binding domain. The IFN-alpha con1 shows greater antiviral activity against HCV replication than the individual IFN-alpha subtypes in vitro [41] as well as in vivo [42]. In our present study, IFN con1 was moderately effective to suppress HCV replication with the IC50 of close to that of IFN-alpha5. However, activation of ISRE and GAS by IFN-alpha con1 seemed to be slight weaker, compared to the other IFN subtypes. Although it might be due to the different definition of units from that of the other IFN-alpha subtypes [30,41], the reportedly strong biological activity of IFN-alpha con1 might also involve pathway other than the Jak-STAT pathway.

Our present results using HCV replicon system have shown that IFN-alpha8 was the strongest to suppress HCV replication among 5 IFN-alpha subtypes 1, 2, 5, 8, and 10. Among clinically used IFN-alpha preparations, natural IFN-alpha preparations contain substantial amounts of IFN-alpha8 [29]. The differential activity shown in this study might direct a spotlight to the drugs, and might propose a hint for more effective IFN drugs used alone or in combination with ribavirin. Taken togather, IFN-alpha8 showed the strongest suppressive effect on in vitro HCV replication. The discrepancy between cellular ISRE responses and the anti-HCV effect implies other pathways other than IFN-activated JAK-STAT pathway. Further investigation of their differential antiviral actions may help elucidating the IFN-mediated cellular defense mechanisms against virus infection.

Acknowledgements

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Analysis of prognostic factors in therapeutic responses to interferon in patients with chronic hepatitis C

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The genotype of hepatitis C virus (HCV) and the amount of HCV RNA are often used to predict the efficacy of interferon (IFN) therapy on chronic hepatitis C. In addition to these factors, there may be several factors related to the host. Therefore, the authors undertook a retrospective study in which physical findings and laboratory data before therapy were evaluated by multiple logistic analysis. Two-hundred and five cases with chronic hepatitis C treated with interferon were analyzed in this study. Sustained virological response was observed with 68 cases. Multiple logistic analysis was performed with 29 explanatory variables including HCV genotype, HCV RNA, IFN types, and total dose, along with gender, age, alcohol consumption, body mass index (BMI), histological findings of liver biopsy, platelet counts, and laboratory data of serum enzymes. Analysis on the factors that correlated well with therapeutic efficacy revealed that genotype 2a, 2b showed higher therapeutic responses than genotype 1b with reference to HCV genotypes, and higher IFN dose or lower HCV RNA levels gave better results. With reference to host factors, higher total protein level, lower levels of BMI, total bilirubin, and aspartate aminotransferase were highly correlated with therapeutic efficacy. HCV genotypes and HCV RNA levels have been already identified as prognostic factors. However, the high correlation values of BMI and the total protein level are new findings. It is suggested that probability estimation of therapeutic effects using the logistic regression equation may be a good tool for predicting therapeutic efficacy of IFN therapy on individual cases. (Translational Research 2006;148:79-86)

Abbreviations: AFP alpha-fetoprotein; ALB albumin; ALP orthophosphoric acid monoester phosphohydrolase; AST aspartate aminotransferase; bDNAp branched DNA probe method; BMI body mass index; BUN blood urea nitrogen; CAH2A chronic active hepatitis, mild; CAH2B chronic active hepatitis, severe; CHE acylcholine acylhydrolase; Cl chloride; CPH chronic persistent hepatitis; CRE creatinine; DB direct bilirubin; GGT gamma glutamyltransferase; HCV hepatitis C virus; HLBI human lymphoblastic interferon; ICG indocyanine green; IFN interferon; K potassium; LAP leucine aminopeptidase; LC liver cirrhosis; LD lactate dehydrogenase; MU mega-unit; Na sodium ions; NR nonresponder; PEG polyethylene glycol; PLT platelet count; SD standard deviation; SE standard error; SVR sustained viral responder; TB total bilirubin; TC total cholesterol; TG triacylglycerol; TP total protein; UA uric acid

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If chronic hepatitis induced by HCV is persistent for a long period of time, there is a high occurrence rate of liver cirrhosis and eventually of hepatocellular carcinoma. There are 1.5 to 2 million carriers of HCV in Japan, and approximately 80% of hepatocellular carcinoma originate from HCV infection.[1] Therefore, therapeutic approaches to chronic hepatitis C are an important issue with respect to prevention of hepatocellular carcinoma.[2] IFNs are one of the mainstream anti-viral therapies to treat chronic hepatitis C, and the factors that determine their therapeutic efficacy include the HCV genotype and the amount of HCV RNA, many of which are attributed to the HCV infection itself.[2–6] As for the therapeutic side of the story, major determinants include the total amount of IFN, therapeutic periods,[7,8] combination therapy with ribavirin,[9,10] and IFN products with PEG modification. [11]

Although factors belonging to the viral and therapeutic aspects can certainly predict therapeutic efficacy in a large portion of chronic hepatitis C, some cases show remarkable SVR despite HCV genotype 1b and high RNA values that ordinarily preclude therapeutic efficacy of IFN. Also, some cases show a poor response despite the HCV genotype 2a and low RNA values that are supposed to be good indicators of therapeutic efficacy. The presence of these cases implies that other factors are related to the host that determine the therapeutic efficacy of IFN against HCV infection. The candidates for host-related factors that may affect the therapeutic efficacy of IFN include gender, age, alcohol uptake, obesity, serum biochemical values, and platelet counts before IFN therapy, which may outline liver function. Therefore, the authors conducted a retrospective study to determine the host-related factors that may influence the therapeutic efficacy of IFN with chronic hepatitis C cases. To predict major prognostic factors out of several factors related to therapeutic efficacy, multiple logistic analysis was used that can analyze contributing factors independent of their mutual interactions. In this study, only the cases treated with IFN alone were analyzed so that the variance in the therapeutic approaches would be minimized, and that prognostic factors related to the host would be more clearly outlined. If the results of this analysis, based on physical findings and laboratory data before therapy, can predict therapeutic efficacy with high probability, it would lead to more efficient IFN therapy and prove the usefulness of clinical laboratory tests.

METHODS

Patients: Two hundred and five cases with chronic hepatitis C treated with IFN at the First Department of Internal Medicine (Digestive Organs), University of Yamanashi, from 1992 to 1996 were analyzed in this study. Of these, there were 155 men with the average age of 43.7 years (SD 9.8 years) and 50 women with the average age of 49.7 years (SD 8.6 years). The total demographic profile is 45.2 9.8 years. All diagnostic and therapeutic procedures were carried out according the principles of the Declaration of Helsinki, and informed consent was obtained from all participants. Furthermore, the study was approved by the review board of the University of Yamanashi. The experimental design of this retrospective study is, in brief, a case control study that enrolls 205 cases of chronic hepatitis C who underwent 24–26 weeks of IFN therapy and was evaluated for its efficacy 6 months after the therapy. Among the prognostic factors including physical findings and laboratory data before therapy, the major factors related to the efficacy of IFN therapy were analyzed with the therapeutic achievement of these 205 patients.

Therapeutic efficacy: The object variable of this study to be analyzed is the therapeutic efficacy of IFN, namely, probability of SVR. The evaluation standard for therapeutic efficacy is negative conversion of HCV-RNA assessed by COBAS AMPLICOR HCV assay (Roche Diagnostics, Switzerland) at some point elapsing more than 6 months after cessation of IFN therapy. Based on this evaluation standard, 68 cases were found to have SVR. The cases without negative conversion of HCV-RNA and with persistent ALT elevation were designated as NRs, and 137 cases in this study fell into this category.

Interferon therapy: With reference to IFN regimens, 166 cases received IFN alpha monotherapy (HLBI, human lymphoblastic interferon; Sumiferon, Sumitomo Pharmaceuticals, Japan), whereas 39 cases underwent IFN alpha-2b monotherapy (Intron A; Schering-Plough, Kenilworth, NJ). With IFN alpha, 6 MU were given daily for the first 2 to 4 weeks, and during the following 22 weeks, 6 MU were given 3 days a week, amounting to the total dose of 480–560 MU. With IFN alpha-2b, 9 MU instead of 6 MU were given with the protocol similar to IFN alpha, amounting to the total dose of 720 to 840 MU.

Explanatory variables: The explanatory variables in this study include HCV genotype and HCV RNA, which have been alleged to be major determinants, and IFN types and total dose. As for those related to the host, 29 factors were analyzed, including gender, age, alcohol consumption, BMI, histological findings of liver biopsy, laboratory data of 19 serum enzymes, and platelet counts. Laboratory tests were measured with fasting blood in the early morning soon after admission into hospital and before the beginning of IFN therapy.

The details of the explanatory variables in this study are as follows: HCV genotypes are classified into genotypes 1 to 6, and subclassified as a, b, c, and so on. The genetic distribution of HCV among Japanese patients are 1b approximately 70%, 2a 20%, and 2b 10%, and there are virtually no other types among Japanese patients.[12] HCV RNA was determined by the branched DNA probe method (bDNAp, Ver. 1.0; Chiron, UAS/ Daiichi Pure Chemicals, Japan) and presented as quantitative values (kMeq/L). Alcoholic consumption was expressed as average daily alcohol intake (g/day, questionnaire-

Table I. Comparison of explanatory variables between SVR and NR groups

Variable	Unit	SVR			NR			P value
		25%	50%	75%	25%	50%	75%	
HCV genotype		1b	2b	2a	1b	1b	1b	0.000
bDNAp	kMeq/L	0.20	0.29	0.83	0.58	1.80	3.81	0.000
IFN type		HLBI	HLBI	HLBI	HLBI	HLBI	HLBI	0.981
IFN dose	MU	516	522	774	516	516	774	0.709
Gender		Male	Male	Male	Male	Male	Female	0.114
Age	years	37	43	53	39	45	52	0.417
Alcohol	g/day	0	25	63	0	25	50	0.544
BMI	Kg/m^2	20.7	22.9	24.7	22.1	24.0	25.8	0.010
Histological type		CAH2A	CAH2A	CAH2A	CPH	CAH2A	CAH2B	0.533
PLT	$10^9/L$	150	188	218	150	182	220	0.856
T P	g/L	73	75	79	70	73	77	0.001
ALB	g/L	41	43	45	40	42	44	0.066
T B	mol/L	10	14	17	12	14	19	0.290
D B	mol/L	3	3	5	3	3	5	0.916
CHE	U/L	262	312	372	261	315	358	0.897
ALP	U/L	133	169	216	134	170	219	0.985
LAP	U/L	48	61	72	49	58	71	0.863
GGT	U/L	33	46	103	30	54	101	0.738
LD	U/L	172	249	305	175	244	316	0.756
AST	U/L	38	52	86	35	52	78	0.673
ALT	U/L	63	100	145	51	82	132	0.090
T G	mmol/L	0.84	1.11	1.52	0.88	1.11	1.41	0.829
T C	mmol/L	3.62	4.09	4.53	3.72	4.19	4.65	0.310
BUN	mmol/L	3.6	4.6	5.4	3.9	5.0	5.7	0.030
CRE	mol/L	53	62	71	53	62	71	0.901
U A	mol/L	280	333	404	274	327	369	0.301
Na	mmol/L	140	141	142	139	140	142	0.036
K	mmol/L	3.9	4.1	4.4	3.9	4.0	4.3	0.517
Cl	mmol/L	101	103	105	101	103	105	0.625

based). Quantitated alcohol intake was checked as those previous to the study period. The histological findings of the liver were classified as CPH, CAH2A, CAH2B, and LC, and these classifications practically corresponded with liver fibrosis stage classifications of F1 to F4, respectively. Serum chemical constituents were analyzed by a 7250 Clinical Analyzer (Hitachi High-Technologies, Japan), and the total protein, serum bilirubin, and enzyme activities were determined by the biuret method and bilirubin-oxidase method,[13,14] and the standardized methods proposed by Japanese Society of Clinical Chemistry,[15] respectively. Platelet counts were measured with a SE9000 Automated Hematology Analyzer (Sysmex, Japan).

Statistics: Statistical analysis was performed using multiple logistic analysis to estimate odds ratios of prognostic factors and to make a prognostic equation of therapeutic effects.[16,17] Briefly, logistic regression equation was estimated with a probability of SVR as object variable and related predictive variables as explanatory variables. All factors were first analyzed in one step analysis, and then a stepwise model was used to select appropriate explanatory variables. A test of the significance of the regression coefficients was performed, and odds ratios of each predictive variables were also estimated. A SPSS Professional Statistics software (SPSS Inc., Japan) was used for computer analysis of all data.

RESULTS

The authors first performed monovariate analysis on all cases. The patients were divided into 2 groups, SVR and NR, and with each group, the values for 25th, 50th (median), and 75th percentile of the explanatory variable are presented in Table I. The data are presented this way, because simple presentation of the data as the means SD could not give an idea as to whether the data assume normal distribution or skewed distribution of continuous variables, and it was not applicable to the categorical variables. The *P*-value for each explanatory variable is listed in the right-most lane, which represents the probability of type I error of the Mann–Whitney *U*-test (the probability of discarding the null hypothesis that there are no differences between 2 groups). The explanatory variables with significant differences between the 2 groups are HCV genotype, HCV-RNA, BMI, TP, BUN, and Na. Most measurement values were the results of standardized methods of

Table II. Multiple logostic analysis of all 29 factors for estimate of the therapeutic efficacy of IFN

Variable	Beta	SE(Beta)	z value	*P* value
HCV genotype	2.288	0.500	4.578	0.0000
bDNAp	0.631	0.198	3.190	0.0014
IFN type	0.053	0.995	0.054	0.9571
IFN dose	0.006	0.003	1.928	0.0539
Gender	0.833	1.156	0.721	0.4712
Age	0.036	0.041	0.875	0.3816
Alcohol	0.112	0.177	0.633	0.5270
BMI	0.238	0.127	1.875	0.0608
Histological type	0.698	0.493	1.417	0.1566
PLT	0.008	0.008	1.087	0.2770
TP	0.305	0.093	3.282	0.0010
ALB	0.051	0.180	0.286	0.7752
TB	0.352	0.132	2.662	0.0078
DB	0.775	0.352	2.203	0.0276
CHE	0.003	0.004	0.793	0.4276
ALP	0.007	0.005	1.449	0.1475
LAP	0.046	0.034	1.353	0.1761
GGT	0.007	0.007	1.060	0.2892
LD	0.003	0.007	0.391	0.6962
AST	0.025	0.017	1.442	0.1494
ALT	0.000	0.009	0.033	0.9737
TG	1.325	0.791	1.676	0.0938
TC	0.697	0.460	1.514	0.1300
BUN	0.360	0.322	1.119	0.2631
CRE	0.013	0.032	0.388	0.6978
UA	0.007	0.007	1.116	0.2645
Na	0.050	0.174	0.289	0.7724
K	0.144	0.937	0.153	0.8782
Cl	0.145	0.127	1.142	0.2533

measurement, and the sample data are considered to represent the range of distribution generally observed in patients with chronic hepatitis C. In addition to the measurement items listed here, AFP, ICG, and type IV collagen 7S, which is a marker of liver fibrosis, were analyzed in a preliminary study. However, as these factors were not measured in all cases analyzed in this study, and furthermore they were found to have no significant correlation with therapeutic efficacy, possibly due to a small number of samples, these data were omitted from this report.

For multivariate analysis of these data, logistic regression analysis was first performed with all 29 factors for prediction of therapeutic efficacy in all patients receiving IFN therapy (Table II). The contribution of each variable to therapeutic efficacy can be estimated by the z value, which is the regression coefficient of each variable (Beta) divided by its standard error [SE-(Beta)], and by the *P* value, which represents the probability of *z* value occurrence. The factors with a high probability of contributing to the therapeutic efficacy thus identified are HCV genotype, HCV RNA, TP, TB, and DB. However, in contrast to expectation, alcohol consumption, IFN type, histological findings of the liver, and platelet counts were not closely correlated with the therapeutic efficacy.

Taking into consideration the preliminary results of the aforementioned analysis with all 29 factors, variable selection in stepwise trials of increment/decrement in the number of variables was repeatedly performed so that reliable and reproducible regression could be attained with the minimal number of variables. This process allowed us to select appropriate explanatory variables and best-fit regression, as shown in Table III. The factors that can best estimate the therapeutic efficacy of IFN are HCV genotype (x_1), HCV RNA (x_2), IFN dose (x_3), BMI (x_4), TP (x_5), TB (x_6), DB (x_7), and AST (x_8). Assessed with *z* and *P* values, HCV genotype, HCV RNA and TP are shown to be most closely correlated with the therapeutic efficacy (*P* 0.1%), followed by IFN dose, BMI, bilirubin, and AST with the *P* values approximately 1%, which is within the level of significance. The logistic regression equation based on these regression coefficients (Beta) to estimate the probability of SVR (p(x)) of IFN therapy is as follows:

$$1 \quad p\,x \quad 1/[1 \quad \exp \quad (\quad 19.006 \quad 2.188\,x_1 \quad 0.554\,x_2 \quad 0.004\,x_3 \quad 0.232\,x_4 \quad 0.246\,x_5 \quad 0.224\,x_6 \quad 0.543\,x_7 \quad 0.014\,x_8) \ ]$$

It should be added that, because the distribution of each variable more closely resembles skew logarithmic normal distribution than normal distribution, the data with each variable in logarithmic transformation were also evaluated with logistic analysis, which produced the results virtually similar to those without variable transformation.

These analytical processes extracted several prognostic factors closely related with the therapeutic efficacy of IFN, as shown in Table III. The authors then evaluated how each of these prognostic factors as a single variable works in the SVR group as compared with the NR group, as shown in Table I. With SVR, HCV genotype with 2a, 2b, 1b in order of power works in favor of therapeutic efficacy, whereas with the NR group, HCV genotype of 1b predominates. Similarly, with the SVR group, there are smaller amounts of HCV RNA, less BMI, and with higher TP values than the NR group (*P* 0.01). However, there are no significant differences between the SVR and NR groups with IFN dose, TB, DB, and AST. On the other hand, with multivariate analysis, the results of which are presented in Table III, all of these factors including IFN dose, TB, DB, and AST are closely related to the therapeutic efficacy of IFN. Thus, the results of factor determina-

Table III. Multiple logistic regression equation for estimate of the therapeutic efficacy of IFN

Degree	Variable	Beta	SE(Beta)	z value	*P* value
x_0		19.006	4.810	3.951	0.00008
x_1	Genotype	2.188	0.397	5.513	0.00000
x_2	bDNAp	0.554	0.164	3.385	0.00071
x_3	IFN dose	0.004	0.002	2.563	0.01037
x_4	BMI	0.232	0.090	2.574	0.01007
x_5	TP	0.246	0.055	4.472	0.00001
x_6	TB	0.224	0.081	2.763	0.00573
x_7	DB	0.543	0.222	2.449	0.01431
x_8	AST	0.014	0.006	2.485	0.01295

Table IV. Odds ratios of prognostic factors for the therapeutic efficacy of IFN

Variable	Change	Odds Ratio	95% Confidence Interval
Genotype	1b to 2b	8.91	4.10–19.40
bDNAp	1.00 kMeq/L	1.74	1.26–2.40
IFN dose	100 MU	1.55	1.11–2.18
BMI	5.0 Kg/m^2	3.19	1.32–7.73
TP	5.0 g/L	3.42	2.00–5.87
TB	9 mol/L	7.51	1.80–31.34
DB	2 mol/L	2.96	1.24–7.07
AST	50 U/L	2.01	1.16–3.48

Example: A case with BMI 2.0 Kg/m^2, TP 5.0 g/L, and TB 9 mol/L would result in 40.85-fold increase in odds ratio.

tion related to IFN therapeutic efficacy differ between single-variate analysis and multivariate analysis.

How each prognostic factor affects the therapeutic efficacy of IFN can be learned from Table IV. The "change" column in Table IV refers to a certain level of increase/decrease in each variable or transition in the categorical variable. These changes can affect the therapeutic efficacy of IFN, irrespective of their reference values. The effect of a particular rate of changes in each variable on IFN therapeutic efficacy is expressed by odds ratios in Table IV, and an 8.91-fold therapeutic efficacy can be expected with HCV genotype of 2b over genotype 1b, 1.74-fold with bDNAp 100 kMeq/L lower, and with IFN dose 100 MU higher, 1.55-fold efficacy can be expected. Similarly, with the host-related prognostic factors, if BMI is 5.0 Kg/m^2 lower, 3.19-fold higher IFN therapeutic efficacy, 3.42-fold with TP 5.0 g/L higher, 7.51-fold with TB 9 mol/L lower, 2.96-fold with DB 2 mol/L higher, and 2.01-fold with AST 50 U/L lower can be expected. These factors are independently related to the therapeutic efficacy of IFN with statistically significant contribution, as shown by the 95% confidential intervals of odds ratios for each variable. Furthermore, the combined effects of several prognostic factors can be expressed as odds ratios. For example, as shown in the lower panel of Table IV, given that HCV genotype, HCV RNA, and IFN dose are the same, if a patient has a profile of BMI 2.0 Kg/m^2, TP 5.0 g/L, TB –9 mol/L, the odds ratio for therapeutic efficacy is 40.85, even though the changes in BMI, TP, and TB are minimal.

$$2 \quad \text{Odds ratio} \quad \exp \quad 0.232 \quad (\ 2.0)$$
$$0.246 \quad 5.0 - 0.224 \quad (\ 9.0) \quad 40.85$$

Thus, logistic analysis employed in this study can be used not only to estimate the prognostic factors, but also to estimate the odds ratios of each factor or multiple factors combined on object variables.

DISCUSSION

Virus-related factors such as HCV genotype, HCV RNA levels, and genetic mutations in NS5A region are alleged to be good markers to predict the effect of anti-viral therapy on chronic hepatitis C.[2–6] The factors related to therapeutic agents include IFN dose, duration of therapy,[7,8] origin of IFN such as those of recombinant IFN versus cell-derived,[18] combined therapy with ribavirin,[9,10] and IFN modified with PEG. [19] Most recently, it is being recognized that the combined therapy of PEG-IFN and ribavirin is the first choice as anti-viral therapy for chronic hepatitis C.[20]

On the other hand, to further augment the efficacy of IFN therapy, it is imperative to investigate the prog-

nostic factors related to recipients with which the therapeutic efficacy of IFN can be predicted. Gender, age, alcohol intake, liver function before therapy, platelet counts, and liver fibrosis have been considered as the host-related factors that determine the IFN therapeutic efficacy. Retrospective studies have disclosed that BMI higher than 30 kg/m^2 negatively affects anti-viral therapy and that it works independently of HCV genotype or liver fibrosis.[21,22]

As described, the factors that modify the efficacy of IFN therapy are multiple, and there seem to be large individual differences in the factors involved and the degrees of contributing factors. To make the issue more complicated, it would take a long time to evaluate the effects of these prognostic factors. With these cases in which the relationship between prognostic factors and therapeutic efficacy is difficult to prove directly, an analytic method is useful that can evaluate in terms of probabilities the contribution of explanatory variables to predict object variables. Thus, in this study, multiple logistic regression analysis is employed that proved extremely useful in analyzing risks of coronary heart diseases in the Framingham study. This analytical method has several advantages over the other methods; it can analyze the data in which explanatory variables include binary variables as well as quantitative data. It can express as odds ratio the contribution of a particular explanatory variable to object variables independently of other interacting explanatory variables. It can calculate the probability of the object occurrence for each case. Thus, multiple logistic regression analysis is a useful and practical tool in analyzing prognostic factors for clinical outcome.

The results of this study in analyzing prognostic factors for the therapeutic efficacy of IFN extracted out of 29 factors several major contributing factors. Those related to HCV include HCV genotype and HCV RNA, and IFN dose is also one major contributing factor. Their correlations with IFN therapeutic efficacy are in good agreement with previous reports. With regard to the host-related factors, the authors found that in addition to BMI, TP, TB, DB, and AST are contributing factors. Particularly, the magnitude of contribution of TP to the overall prediction of IFN therapeutic effect is higher than that of HCV RNA or BMI, and it is also to be noted that this report is the first to propose TP as a major prognostic factor in IFN therapeutic efficacy, to the best of the authors' knowledge. A previous paper suggested that GGT is an independent predictive factor of SVR,[23] although in this study, GGT was not a significant indicator of SVR. Their study dealt with a group of patients treated with IFN and ribavirin, and it is conceivable that the differences in the composition of patients and the modes of therapy are responsible for the discrepancy.

The authors then sought to elucidate why TP among other host-related prognostic factors was most closely correlated with the therapeutic efficacy of IFN. It is widely accepted that the more intact hepatic cells remain, the more effective is IFN therapy. In this line of reasoning, TP and ALB represents the protein producing activity of the liver, and they are expected to positively correlate with the efficacy of IFN therapy. There is no clear explanation for the superior predictability of TP to ALB in IFN therapeutic efficacy, as shown in this study. It would be of interest to explore contributing factors (proteins) that make TP more superior to ALB in predicting the therapeutic efficacy of IFN in further studies. AST and bilirubin levels represent the level of hepatic damage or impairment of its function, and they should be negatively correlated with the IFN therapeutic efficacy. With higher BMI index, there are more lipids in hepatocytes, and this is assumed to absorb IFN and to interfere with the signal transduction pathways related to IFN receptors. BMI may be also an index of underlying liver fibrosis that antagonizes the action of IFN.[21,22]

To further explore the cause for TP superiority over other prognostic factors, the data were divided into 3 groups based on HCV genotype (Table V). With HCV genotypes of 2a and 2b, which are comparatively good responders to IFN therapy, TP is positively, and BMI, bilirubin, and AST are negatively, correlated with IFN therapy. As the number of cases with genotype 2a and 2b are small, the authors did not perform multivariate analysis with these groups alone. With HCV genotype 1b, NRs predominated over SVR (106 vs 25). In contrast to HCV genotype 2a and 2b, there were only small differences between SVR and NR with regard to BMI, bilirubin in this group, and with AST, SVR had a tendency to have higher AST values than NR in the HCV genotype 1b group. On the other hand, TP consistently gave higher values with SVR, as in the case of HCV genotype 2a and 2b. Although the reasons BMI, bilirubin, or AST are not related to the IFN therapeutic efficacy with HCV genotype 1b remain elusive, it is of interest that TP can serve as a good predictive factor even with this group of patients. Based on these findings, the authors suggest that TP is judged to be the prognostic factor most closely correlated with the therapeutic efficacy of IFN in logistic regression analysis of this study, because it can consistently serve as a good marker for IFN efficacy, regardless of HCV genotype.

The logistic regression equation determined in this study can be used to estimate the therapeutic efficacy of IFN for each case. The authors postulated 4 cases in which the virus- and IFN-related factors are in various