

2) BMS-790052(NS5A阻害薬)+BMS-650032(プロテアーゼ阻害薬)(プリストルマイヤーズ・スクイブ)

genotype 1のPEG-IFN+RBV治療無効例に対するNS5A阻害薬BMS-790052とプロテアーゼ阻害薬BMS-650032による臨床第II相試験が行われた。BMS-790052(60mg/日)+BMS-650032(1,200mg/日)による2剤併用療法(11例)あるいはPEG-IFN+RBVを加えた4剤併用療法(10例)の24週間の比較試験である。

①BB24: SVRは36%(4/11)

②PRBB24: SVRは90%(9/10)

との成績であった。

興味深いことに、2剤併用療法でウイルス学的ブレイクスルーの認められた症例は全例genotype 1aであり、genotype 1aでのSVRが22%(2/9)であるのに対し、genotype 1bでのSVRは症例数が少ないながらも100%(2/2)であった。また、ウイルス学的ブレイクスルーの認められた症例では、耐性変異について検討したところ、両剤に対する耐性が出現していた。それでも、PEG-IFN+RBVなしで実際にウイルス駆除が可能であることが示された意義は大きい。

我が国でも同様の試験が行われ、2011年11月の米国肝臓学会でその成績が報告された。genotype 1bのPEG-IFN+RBV治療無効例10例に対する2剤併用療法の試験で、SVRは90%(9/10)であったが、脱落例もウイルス駆除を達成しており、実質100%のウイルス駆除を達成している。

3) テラプレビル(プロテアーゼ阻害薬)+VX-222(ポリメラーゼ阻害薬)(Vertex)

2010年12月、Vertexはテラプレビルとポリメラーゼ阻害薬VX-222併用臨床第II相試験の一部中止を発表した。ウイルス再燃に関する中止ルールに合致したことにより、テラプレビルとVX-222の2剤治療が中止された。

4) GS-9256(プロテアーゼ阻害薬)+tegobuvir(GS-9190, ポリメラーゼ阻害薬)(Gilead)

genotype 1, 未治療のC型慢性肝炎患者に対

し、プロテアーゼ阻害薬GS-9256を75mg, 1日2回とポリメラーゼ阻害薬tegobuvirを40mg, 1日2回投与のみ、それらにRBVを加えたもの、それにPEG-IFNを加えた3つのアームの試験が行われている。

①GT4/PR44: rapid virological response (RVR)は7%(1/15)

②RGT4/PR44: RVRは38%(5/13)

③PRGT4/PR44: RVRは100%(15/15)

との成績が得られている。

5. 宿主細胞標的薬

宿主因子を標的にした薬剤の開発が行われている。宿主因子を標的にする利点は、耐性が生じにくいことと、どのgenotypeでも有効性が期待されることである。宿主細胞標的薬ではサイクロフィリン阻害薬の開発が最も進んでいる。

1) DEBIO-025(Alisporivir, DEBIO Pharm)

サイクロスポリンに抗HCV活性があることが報告され、その標的はサイクロフィリンであることが明らかになった。サイクロスポリンは強力な免疫抑制剤であるが、抗HCV効果は免疫抑制効果によらないことが示された。現在開発中のDEBIO-025はDEBIO Pharm社による免疫抑制作用のないサイクロフィリン阻害薬である⁹⁾。臨床第II相試験が行われており、genotype 1あるいは4の未治療C型慢性肝炎患者に対し、600-1,000mg/日のDEBIO-025とPEG-IFN+RBVを組み合わせ、4週で4.6-4.8 log₁₀ IU/mLのHCV RNAの低下を認めている。

6. その他

1) Zalbin/joulferon/albuferon(albinterferon α -2b)(ノバルティス)

IFN α 2bにアルブミンを結合したalbuferonは、PEG-IFNよりも長期間安定であり、2-4週間に1度の投与でPEG-IFNに匹敵する治療効果が得られるとのことで、大変に期待されていたIFN製剤である。しかしながら、2010年10月、ノバルティスは、Human Genome Sciencesと提携のもとで開発してきたalbuferonの開発

中止を発表した。安全性への懸念(特に間質性肺炎)を指摘されたためである。

2) PEG-IFN λ (ブリストルマイヤーズ・スクイブ)

PEG-IFN λ +RBV併用療法で、PEG-IFN α 2aを上回る治療効果が得られている。PEG-IFN λ はPEG-IFN α 2a同様、interferon stimulated genes(ISGs)を誘導するが、異なるレセプターを利用しており、PEG-IFN λ のレセプターを発現している組織が限られているために、PEG-IFN α 2aよりも全身的副作用が少ない。

PEG-IFN λ の120 μ g/180 μ g/240 μ gと体重換算のRBVを、genotype 1および4に対しては48週間投与、genotype 2および3に対しては24週間投与を行う試験が行われている。genotype 1および4に対する試験結果を示す。

- ① PR48(SOC): cEVRは37.9%
- ② PEG-IFN λ (120 μ g)+RBV48: cEVRは55.0%
- ③ PEG-IFN λ (180 μ g)+RBV48: cEVRは55.9%
- ④ PEG-IFN λ (240 μ g)+RBV48: cEVRは56.3%

との成績であり、有意にPEG-IFN α 2aより高率にcEVRが得られていた。

現在までの試験結果をまとめると、

- ・ genotype 1, 2, 3, 4におけるPEG-IFN λ 180/240 μ gのRVRはPEG-IFN α 2aより優れていた。

- ・ genotype 1, 4におけるPEG-IFN λ 全用量のcEVRはPEG-IFN α 2aより優れていた。
- ・ genotype 2, 3におけるPEG-IFN λ 全用量のcEVRはPEG-IFN α 2aと同様であった。
- ・ IL28Bにかかわらず抗ウイルス効果はPEG-IFN λ でPEG-IFN α 2aより増加した。
- ・ PEG-IFN λ 全用量でIFNおよびRBVの減量率はPEG-IFN α 2aより減少した。
- ・ PEG-IFN λ では血球系の異常は少なかった。
- ・ PEG-IFN λ 240 μ gでのみAST/ALTの上昇が認められた。
- ・ PEG-IFN λ での高ビリルビン血症4例は中止により全例回復した。

となる。

前述のように、発熱、関節痛、筋肉痛、悪寒、発疹などの副作用は、PEG-IFN α 2aに比しPEG-IFN λ では非常に少ない。また、PEG-IFN λ そのものの減量も少ないが、RBVの減量も少なく、cEVRの増加に貢献していると思われる。

おわりに

我が国のC型肝炎患者は高齢化してきており、IFN治療の適応とならないことも多い。そのため、将来的に作用機序の異なる複数の経口薬の併用療法にてウイルス駆除が可能となることが強く期待される。

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Short Communication

Gene expression profile of Li23, a new human hepatoma cell line that enables robust hepatitis C virus replication: Comparison with HuH-7 and other hepatic cell lines

Kyoko Mori,* Masanori Ikeda, Yasuo Ariumi and Nobuyuki Kato*

Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

Aim: Human hepatoma cell line HuH-7-derived cells are currently the only cell culture system used for robust hepatitis C virus (HCV) replication. We recently found a new human hepatoma cell line, Li23, that enables robust HCV replication. Although both cell lines had similar liver-specific expression profiles, the overall profile of Li23 seemed to differ considerably from that of HuH-7. To understand this difference, the expression profile of Li23 cells was further characterized by a comparison with that of HuH-7 cells.

Methods: cDNA microarray analysis using Li23 and HuH-7 cells was performed. Li23-derived ORL8c cells and HuH-7-derived RSc cells, in which HCV could infect and efficiently replicate, were also used for the microarray analysis. For the comparative analysis by reverse transcription polymerase chain reaction (RT-PCR), human hepatoma cell lines (HuH-6, HepG2, HLE, HLF and PLC/PRF/5) and immortalized hepatocyte cell line (PH5CH8) were also used.

Results: Microarray analysis of Li23 versus HuH-7 cells selected 80 probes to represent highly expressed genes that have ratios of more than 30 (Li23/HuH-7) or 20 (HuH-7/Li23). Among them, 17 known genes were picked up for further analysis. The expression levels of most of these genes in Li23 and HuH-7 cells were retained in ORL8c and RSc cells, respectively. Comparative analysis by RT-PCR using several other hepatic cell lines resulted in the classification of 17 genes into three types, and identified three genes showing Li23-specific expression profiles.

Conclusion: Li23 is a new hepatoma cell line whose expression profile is distinct from those of frequently used hepatic cell lines.

Key words: hepatitis C virus, hepatoma cell line, HuH-7, Li23, microarray

INTRODUCTION

HuH-7, A HUMAN hepatoma cell line,¹ is frequently used in the research of hepatitis C virus (HCV), since an HCV replicon system enabling HCV subgenomic RNA replication was developed using HuH-7 cells.² Even with the use of an efficient HCV production system developed in 2005,³ HuH-7-derived cells are still used as the only cell line for persistent HCV production systems.

We previously developed HCV replicon systems^{4,5} and an HCV production system⁶ using HuH-7-derived cells. Furthermore, we recently found a new human hepatoma cell line, Li23, that enables robust HCV RNA replication and persistent HCV production.⁷ In that study, using microarray analysis, we excluded the possibility that the obtained Li23-derived cells were derived from contamination of HuH-7-derived cells used for HCV replication.⁷ In addition, we noticed that the gene expression profile of Li23 cells seemed considerably different from that of HuH-7 cells. Therefore, we assumed that the Li23 cell line possesses a unique expression profile among widely used human hepatoma cell lines. To evaluate this assumption, we further characterized the expression profile of Li23 cells by comparing it with those of other human hepatoma cell lines, including HuH-7,¹ HuH-6,⁸ HepG2,⁹ HLE,¹⁰ HLF¹⁰ and PLC/PRF/5.¹¹ Human immortalized hepatocyte cell line

Correspondence: Professor Nobuyuki Kato, Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8558, Japan. Email: nkato@md.okayama-u.ac.jp

*These authors contributed equally to this work.

Received 21 July 2010; revision 16 August 2010; accepted 17 August 2010.

PH5CH8¹² was also used for the comparison. Here, we show that the Li23 cell line possesses a distinct expression profile among hepatic cell lines.

METHODS

Cell culture

HUH-7, HUH-6, HEPG2, HLE, HLF and PLC/PRF/5 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Li23 and PH5CH8 cells were maintained as described previously.⁷ Cured cells (Li23-derived ORL8c and HuH-7-derived RSc), from which the HCV RNA had been eliminated by interferon (IFN) treatment, were also maintained as described previously.⁷

cDNA microarray analysis

Li23, ORL8c, HuH-7 and RSc cells (1×10^6 each) were plated onto 10-cm diameter dishes and cultured for 2 days. Total RNA from these cells were prepared using the RNeasy extraction kit (QIAGEN, Hilden, Germany). cDNA microarray analysis was performed according to the methods described previously.⁷ Differentially expressed genes were selected by comparing the arrays from Li23 and HuH-7 cells. The selected genes were further compared with the array from ORL8c or RSc cells.

Reverse transcription polymerase chain reaction

Reverse transcription polymerase chain reaction (RT-PCR) was performed to detect cellular mRNA as

described previously.¹³ Briefly, total RNA (2 μ g) was reverse-transcribed with M-MLV reverse transcriptase (Invitrogen, San Diego, CA, USA) using an oligo dT primer (Invitrogen) according to the manufacturer's protocol. One-tenth of the synthesized cDNA was used for PCR. The primers arranged for this study are listed in Table 1. In addition, we used primer sets for New York esophageal squamous cell carcinoma 1 (NY-ESO-1), β -defensin-1 (DEFB1), lectin, galactoside-binding, soluble 3 (LGALS3)/Galectin-3, melanoma-specific antigen family A6 (MAGEA6), UDP glycosyltransferase 2 family polypeptide B4 (UGT2B4), transmembrane 4 superfamily member 3 (TM4SF3), insulin-like growth factor binding protein 2 (IGFBP2), arylacetamide deacetylase (AADAC), albumin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as described previously.⁷

RESULTS

Genes showing pronounced differences in gene expression between Li23- and HuH-7-derived cells

WE RECENTLY ESTABLISHED several Li23-derived cell lines showing robust HCV RNA replication.⁷ In convenient microarray analysis using these cell lines, we noticed that the gene expression profile of Li23 cells differed considerably from that of HuH-7 cells, and that several genes, including cancer antigens such as NY-ESO-1 and MAGEA6, were highly expressed in Li23 cells but were not expressed in HuH-7 cells.⁷ However, it

Table 1 Primers used for reverse transcription polymerase chain reaction analysis

Gene (accession no.)	Direction	Nucleotide sequence (5'-3')	Products (bp)
Cancer antigen 45, A5 (CT45A5); NM_001007551	Forward	TGGAGATGACCTAGAATGCAG	218
	Reverse	CTCGTCTCATAACATCTTGCTG	
Four-and-a-half LIM domain 1 (FHL1; NM_001449)	Forward	GGAATCACTTACCAGGATCAG	243
	Reverse	TTTGCAGTGAAGCAGTAGTC	
Thymosin β 4, X-linked (TMSB4X; NM_021109)	Forward	ACCAGACTTCGCTCGTACTC	179
	Reverse	TCGCCTGCTTGCTTCTCCTG	
Lectin, galactoside-binding, soluble 1 (LGALS1; NM_002305)	Forward	CAACACCATCGTGTGCAACAG	253
	Reverse	CAGCTGCCATGTAGTTGATGG	
Interferon-induced transmembrane protein 2 (IFITM2; NM_006435)	Forward	CCTCTTCATGAACACCTGCTG	184
	Reverse	CACTGGGATGATGATGAGCAG	
Apolipoproteins A1 (APOA1; X02162)	Forward	ACTGTGTACGTGGATGTGCTC	273
	Reverse	CTTCTTCTGGAAGTCGTCCAG	
α -2-HS-glycoprotein (AHSG; NM_001622)	Forward	AACCGAACTGCCGATGATCCAG	248
	Reverse	TTCGACAGCATGCTCCTTCAG	
Gap junction protein- α 1 (GJA1; NM_000165)	Forward	CATCTTCATGCTGGTGGTGTC	253
	Reverse	GTTTCTGTCCAGTAACCAG	

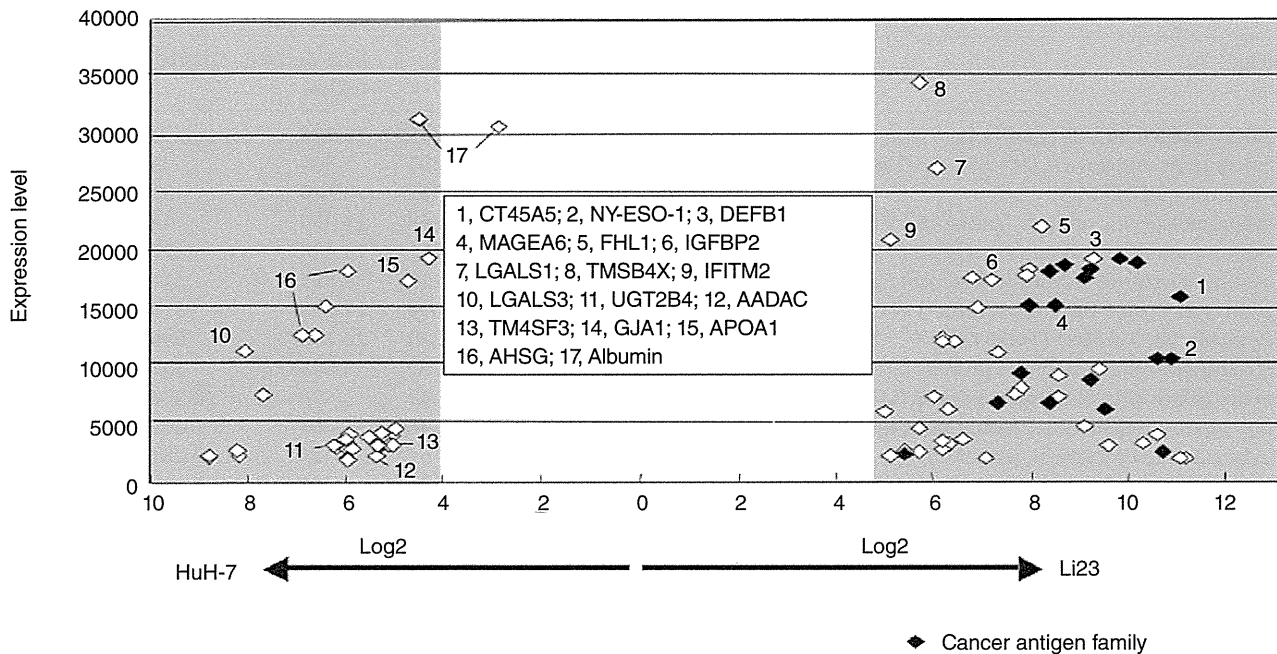


Figure 1 Genes showing pronounced differences in gene expression between Li23 and HuH-7 cells. The probes showing expression levels of more than 2000 and ratios of more than 30 (Li23/HuH-7) or 20 (HuH-7/Li23) are presented.

is unclear whether the expression profiles of these genes are characteristics of Li23 cells.

To clarify this point, comprehensive microarray analysis using Li23 and HuH-7 cells was performed. This revealed 4119 and 3570 probes whose expression levels were upregulated and downregulated at ratios of more than 2 and less than 0.5 in Li23 versus HuH-7 cells, respectively. From among these probes, we selected those showing ratios of more than 30 (Li23/HuH-7) and 20 (HuH-7/Li23), and further selected the probes showing expression levels of more than 2000 (actual value of measurement). By these selections, 80 probes were assigned (Fig. 1). The most distinguishing characteristic of the comparison is that the cancer antigen family (18 probes) was highly expressed in Li23 cells but was not highly expressed in HuH-7 cells (Fig. 1). From these probes, 14 known genes showing expression levels above 10 000 (#1–10 and #14–17 in Fig. 1) and three additional known genes (#11–13 in Fig. 1) were chosen as representative genes for further analysis.

Regarding the total of 17 genes, the expression levels in Li23 versus ORL8c or HuH-7 versus RSc were compared. The expression levels of most of the 17 genes were maintained between Li23 and ORL8c cells or between HuH-7 and RSc cells (Table 2). These results indicate that ORL8c and RSc cells retained the charac-

teristics of parent Li23 and HuH-7 cells, respectively. However, it was notable that the expression levels of apolipoprotein A1 (APOA1), α -2-HS-glycoprotein (AHSG), and albumin were significantly higher in ORL8c cells than in Li23 cells, suggesting that ORL8c is selected as a specific clone from Li23 cell populations.

Expression profiles of representative genes whose expression levels showed drastic differences between Li23 and HuH-7 cells among human hepatic cell lines

Regarding the 17 genes selected above, we performed comparative analyses by RT-PCR using Li23, HuH-7, HuH-6, HepG2, HLE, HLF, PLC/PRF/5 and PH5CH8 cells in order to clarify whether or not these genes exhibit Li23-specific expression profiles. The results of the RT-PCR performed after optimization of PCR conditions in each gene resulted in the classification of the 17 genes into three types (A, B and C in Fig. 2). NY-ESO-1 and DEFB1 (high expression in Li23 only), and LGALS3/Galectin-3 (no expression in Li23 only) belonged to type A, which showed a Li23-specific feature. Type B showed that the expression levels in Li23, HLE, HLF, PLC/PRF/5 and/or PH5CH8 cells were greatly higher or lower than those in HuH-7, HuH-6 and HepG2 cells. Type B consisted of cancer antigen 45, A5

Table 2 Representative genes showing pronounced differences in gene expression between Li23 and HuH-7 cells

Gene	Accession no.	Li23	Li23-derived ORL8c	HuH-7	HuH-7-derived RSc
Cancer antigen 45, A5 (CT45A5)	NM_001007551	15 857†	10 508	8	23
Cancer testis antigen 1A (NY-ESO-1/CTAG1A)	U87459	9 005	5 503	5	8
β -Defensin-1 (DEFB1)	U73945	18 311	8 326	31	7
Melanoma-specific antigen family A6 (MAGEA6)	U10691	15 168	17 050	42	35
Four-and-a-half LIM domain 1 (FHL1)	NM_001449	21 851	13 428	77	79
Insulin-like growth factor binding protein 2 (IGFBP2)	NM_000597	17 429	8 931	117	13
Lectin, galactoside-binding, soluble 1 (LGALS1)	NM_002305	26 694	27 098	379	11
Thymosin β 4, X-linked (TMSB4X)	NM_021109	34 273	26 199	648	307
IFN-induced transmembrane protein 2 (IFITM2)	NM_006435	20 762	9 645	595	637
Lectin, galactoside-binding, soluble 3 (LGALS3/Galectin 3)	BC001120	41	70	10 973	6 020
UDP glycosyltransferase 2 family polypeptide B4 (UGT2B4)	NM_021139	40	57	2 863	7 546
Arylacetamide deacetylase (AADAC)	NM_001086	57	73	2 282	4 746
Transmembrane 4 superfamily member 3 (TM4SF3)	NM_004616	95	51	3 220	1 265
Gap junction protein- α 43 kDa (GJA1)	NM_000165	951	2	19 090	19 485
Apolipoprotein A1 (APOA1)	X02162	673	7 230	16 920	15 202
α -2-HS-glycoprotein (AHSG)	NM_001622	308	6 373	18 436	26 000
Albumin	AF116645	4 304	30 111	30 234	33 140
	D16931	1 387	23 615	30 668	39 144

†Signal intensity in human genome U133 Plus 2.0 array.

(CT45A5), MAGEA6, four-and-a-half LIM domains 1 (FHL1), Thymosin B4, X-linked (TMSB4X), lectin, galactoside-binding, soluble 1 (LGALS1) and IFN-induced transmembrane protein 2 (IFITM2) – all of which were highly expressed in Li23 cells – and APOA1, AHSG and UGT2B4, which were highly expressed in HuH-7 cells. The remaining five genes were assigned to type C and showed more complex expression profiles (Fig. 2). For instance, Gap junction protein- α 43 kDa (GJA1) expression was observed in HuH-7, HLE, HLF, PLC/PRE/5 and PH5CH8 cell lines, but not in Li23, HuH-6 or HepG2 cell lines. In addition, IGFBP2 expression was observed in Li23, HuH-6 and PH5CH8 cell lines, but not in the other cell lines. Together, these results indicate that the Li23 cell line possesses a distinct expression profile among frequently used hepatic cell lines.

DISCUSSION

IN THIS STUDY, we assigned 17 known genes that showed drastic differences between Li23 and HuH-7 cells, and classified the expression profiles of these genes into at least three types among frequently used hepatic cell lines. Three genes (NY-ESO-1, DEFB1 and LGALS3/Galectin-3) were identified as the representative showing Li23-specific expression.

NY-ESO-1 is a well-characterized cancer-testis antigen (CTAG) that appears to be the most immunogenic CTAG known to date.¹⁴ NY-ESO-1 is expressed in malignant tumors such as melanoma, lung carcinoma and bladder cancer, which are called “CTAG-rich” tumor types, but are expressed solely in the testis among normal adult tissues.¹⁵ Because a spontaneous immune response to NY-ESO-1 is frequently observed in patients with malignant tumors including hepatocellular carcinoma,¹⁶ cancer vaccine trials based on NY-ESO-1 are currently underway.¹⁵ However, the biological role of NY-ESO-1 in both tumors and testis remains poorly understood. Accordingly, the Li23 cell line may be useful for the study of the biological role of NY-ESO-1.

Human defensins, which are small cationic peptides produced by neutrophils and epithelial cells, form two genetically distinct subfamilies, α -defensin and β -defensin. DEFB1, identified in this study, is one of six members belonging to β -defensins and appears to be involved in the antimicrobial defense of the epithelia of surfaces.^{16,17} Although α -defensins consisting of six members are known to be expressed in a variety of tumors, DEFB1 is downregulated in some tumor types in which it could behave as a tumor suppressor protein.¹⁸ Our study revealed that except DEFB1 in Li23 cells, no α - or β -defensin members were expressed in the

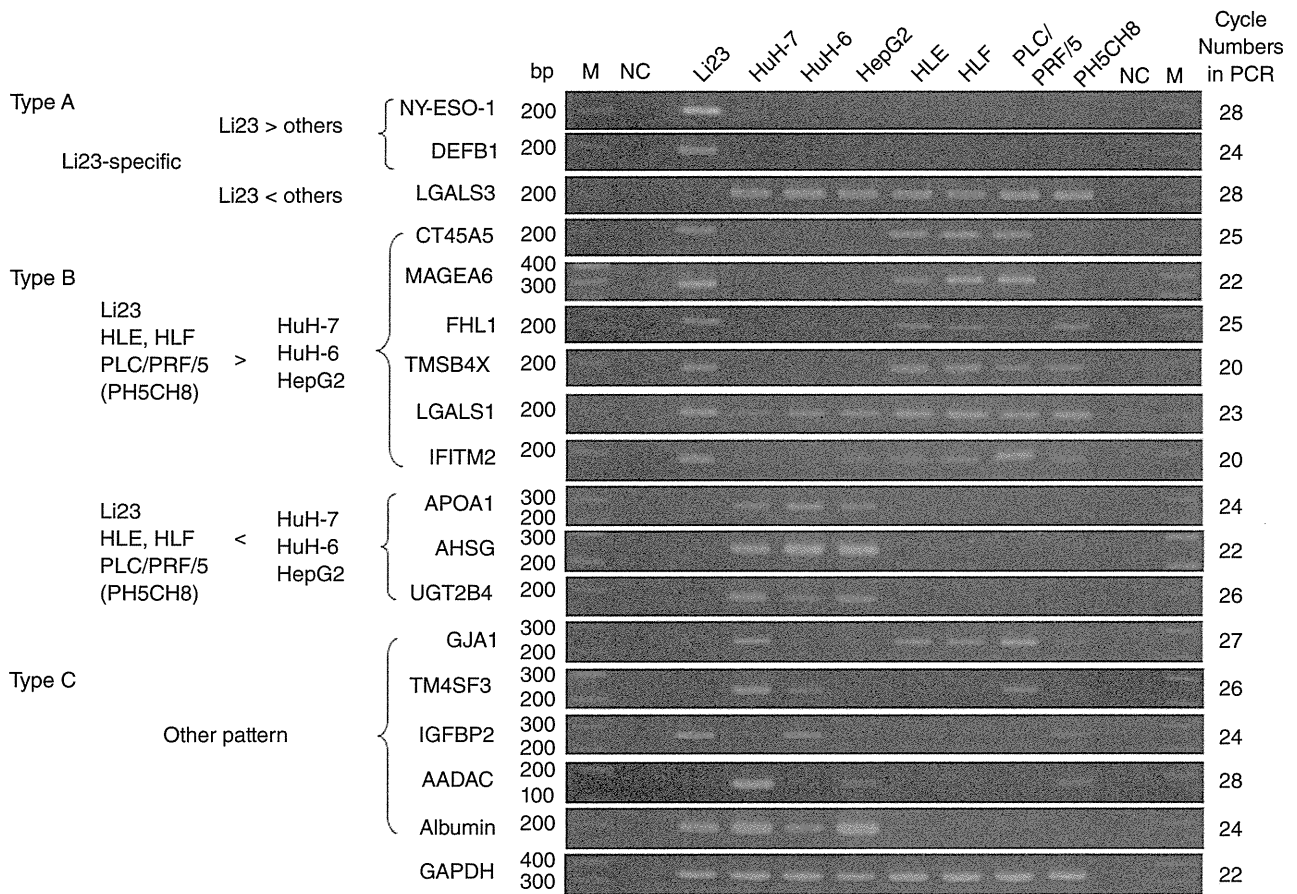


Figure 2 Expression profiles of representative genes, whose expression levels showed drastic differences between Li23 and HuH-7 cells, among human hepatic cell lines. Reverse transcription polymerase chain reaction (RT-PCR) analysis was performed as described in Methods. PCR products were detected by staining with ethidium bromide after separation by electrophoresis on 3% agarose gels.

hepatic cell lines tested in this study (data not shown). Because the molecular mechanism underlying DEFB1 expression or its role in oncogenesis remains to be clarified, Li23 cells may be useful for a study like that.

LGALS3/Galectin-3 is the most studied member of the galectin family, which is characterized by specific binding of β -galactosides through the carbohydrate-recognition domain.¹⁹ LGALS3/Galectin-3 is ubiquitously expressed in numerous cell and tissue types; it is located in both nuclei and cytoplasm, and is secreted through a non-classical pathway. To date, LGALS3/Galectin-3 was found to be involved in many regulations including development, immune reaction, tumorigenesis, and tumor growth and metastasis.^{19,20} Indeed, the overexpression of LGALS3/Galectin-3 in cirrhotic and hepatocellular carcinoma has also been reported.²¹ In such situations, the absence of LGALS3/

Galectin-3 expression in the Li23 cell line is a unique feature among hepatic cell lines, which show high expression levels. Accordingly, the Li23 cell line might be useful as a LGALS3/Galectin-3-null cell line for various studies including those on tumor growth and metastasis.

Although we identified Li23-specific genes showing distinct expression levels among hepatic cell lines examined, microarray analysis revealed that the expression profiles of Li23 and HuH-7 cells, both of which possess an environment for robust HCV replication, differed considerably. Accordingly, such differences may affect the properties or multiplications of HCV, such as susceptibility to anti-HCV reagents, the mutation rate of the HCV genome and the efficiency of HCV replication. Further comparative analysis using Li23 and HuH-7 cells will help to resolve these uncertain subjects.

ACKNOWLEDGMENTS

WE THANK NAOKO Kawahara for her technical assistance. This work was supported by a Grant-in-Aid for research on hepatitis from the Ministry of Health, Labor and Welfare of Japan. K. M. was supported by a Research Fellowship from the Japan Society for Promotion of Science for Young Scientists.

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BASIC STUDIES

Amino acid substitutions of hepatitis C virus core protein are not associated with intracellular antiviral response to interferon- α *in vitro*

Fusao Ikeda^{1,2,3}, Hiromichi Dansako¹, Go Nishimura¹, Kyoko Mori¹, Yoshinari Kawai^{1,2}, Yasuo Ariumi¹, Yasuhiro Miyake^{2,3}, Akinobu Takaki², Kazuhiro Nouse^{2,3}, Yoshiaki Iwasaki², Masanori Ikeda¹, Nobuyuki Kato¹ and Kazuhide Yamamoto^{2,3}

1 Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan

2 Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan

3 Department of Molecular Hepatology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

Keywords

antiviral activity – HCV core – hepatitis C virus – interferon

Correspondence

Fusao Ikeda, MD, Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1, Shikata-cho, Okayama 700-8558, Japan
Tel: +81 86 235 7219
Fax: +81 86 225 5991
e-mail: fiked@md.okayama-u.ac.jp

Received 12 March 2010

Accepted 23 May 2010

DOI:10.1111/j.1478-3231.2010.02299.x

Abstract

Background: Studies on patients with hepatitis C virus (HCV) of genotype 1b have suggested that amino acids (aa) 70 and/or 91 of the HCV core protein affect the outcome of interferon (IFN)- α and ribavirin (RBV) therapy, although there are no clear supporting data *in vitro*. **Aims:** This study was designed to determine the differences among the antiviral activities of HCV core proteins with various substitutions at aa70 and/or aa91. **Methods:** The retroviral vectors expressing the HCV core proteins with substitutions of arginine/leucine, arginine/methionine, glutamine/leucine or glutamine/methionine at aa70/aa91 were transiently transfected or stably transduced into an immortalized hepatocyte line (PH5CH8), hepatoma cell lines and an HCV-RNA replicating cell line (sOR) to evaluate antiviral responses to IFN- α or IFN- α /RBV. Sequence analysis was performed using genome-length HCV-RNA replicating cells (OR6 and AH1) to evaluate HCV core mutations during IFN- α treatment. **Results:** The promoter activity levels of IFN-stimulated genes in the transiently transfected cells or the mRNA levels of 2'-5'-oligoadenylate synthetase in the stably transduced PH5CH8 cells were not associated with the HCV core aa70 and/or aa91 substitutions during IFN- α treatment. Antiviral responses to IFN- α or IFN- α /RBV treatment were enhanced in sOR cells stably transduced with the HCV core, although there were no differences in antiviral responses among the cells expressing different core types. Sequence analysis showed no aa mutations after IFN- α treatment. **Conclusions:** Antiviral activities were enhanced by HCV core transduction, but they were not associated with the HCV core aa70 and/or aa91 substitutions by *in vitro* analysis.

Hepatitis C virus (HCV) infection causes chronic hepatitis, and may progress to cirrhosis and hepatocellular carcinoma. More than 170 million people worldwide are infected with HCV, creating a serious global health problem (1, 2). Interferon (IFN)- α is widely used in the treatment of patients with chronic hepatitis C, and the current combination treatment with pegylated IFN- α and ribavirin (RBV) has improved the sustained virological response, and has a success rate of more than 50% (3). Despite this therapeutic success rate, however, there are still non-viral responders (NVR) to IFN- α treatment. High viral load and genotype 1 of HCV are major viral causes of IFN- α resistance. For patients with HCV genotype 1, variations in the amino acid (aa) sequence of the IFN sensitivity-determining region (ISDR) (4) and

IFN/RBV resistance-determining region (IRRDR) (5) in the non-structural 5A region have also been reported as important predictors of therapeutic outcomes.

Recent studies on the virological features of HCV patients that are most predictive of NVR to IFN- α /RBV therapy (6, 7) proposed that HCV core protein aa70 and/or aa91 substitutions were independent and significant factors for therapeutic outcomes. In particular, substitutions of arginine by glutamine at aa70 and/or of leucine by methionine at aa91 were common in NVR. Patients with the HCV core aa70 substitutions often had a slow or no decrease in HCV-RNA levels during the early phase of IFN- α treatment (6–9). A previous report evaluating HCV dynamics during IFN- α therapy described a biphasic kinetic pattern of HCV-RNA decline, and the viral

decrease in the first phase was believed to be dependent on the direct effect of IFN- α on infected targets (10). We hypothesized that the types of HCV core proteins might alter the antiviral environment in the infected hepatocytes, and thus the aim of this study was to determine the difference in antiviral effects between HCV core proteins with various aa70 and aa91 substitutions.

Methods

Cell cultures

A non-neoplastic immortalized human hepatocyte line (PH5CH8) and hepatoma cell lines (HepG2, HuH-7 and Li23) were used to evaluate antiviral response, as described previously (11–13). We also used sOR cells harbouring subgenomic HCV-RNA derived from an HCV-O strain (genotype 1b) with Neo and *Renilla* luciferase genes (14). A schematic of the gene organization of sOR cells is shown in Figure 1a. All the cell lines used in this study were reported to possess the normal IFN signalling pathway (13–16). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, streptomycin and 0.3 mg/ml of G418 (Geneticin; Invitrogen, Carlsbad, CA, USA).

Plasmid constructions

Four retrovirus pCX4bsr vectors (17) expressing HCV core proteins were constructed, whose aa sequences were identical to the consensus sequences of the HCV core protein from the HCV-O strain encoding aa1–191, except for arginine at aa70/leucine at aa91 for CoreR70L91, glutamine at aa70/leucine at aa91 for CoreQ70L91, glutamine at aa70/methionine at aa91 for CoreQ70M91 and arginine at aa70/methionine at aa91

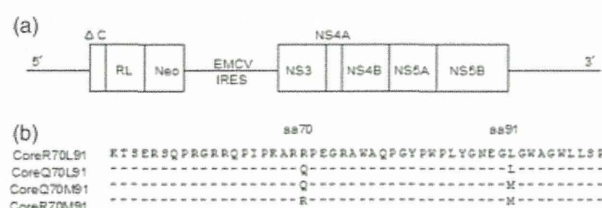


Fig. 1. Schema of subgenomic hepatitis C virus (HCV) RNA and partial aa sequences of the HCV core in the constructed retroviral vectors. (a) A schematic of the gene organization of subgenomic HCV replicon RNA. RL, *Renilla* luciferase; Neo, neomycin phosphotransferase; EMCV IRES, encephalomyocarditis virus internal ribosome entry site. (b) The partial aa sequences of the HCV core protein encoded in the constructed vectors. Four retrovirus pCX4bsr vectors expressing HCV core proteins were constructed, whose aa sequences were identical to the consensus sequences of the HCV core from HCV-O strain encoding aa1–191, except for arginine at aa70/leucine at aa91 for CoreR70L91, glutamine at aa70/leucine at aa91 for CoreQ70L91, glutamine at aa70/methionine at aa91 for CoreQ70M91 and arginine at aa70/methionine at aa91 for CoreR70M91.

for CoreR70M91, as shown in Figure 1b. The two types of HCV core protein, CoreQ70L91 and CoreR70L91, were obtained from cells infected with serum HCV-O in a study of the dynamics of HCV populations during culture (18). CoreQ70M91 and CoreR70M91 were constructed from CoreQ70L91 and CoreR70L91, respectively, by PCR mutagenesis with primers containing base alterations. The sequences of these inserts were confirmed by Big Dye termination cycle sequencing using an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Preparation of PH5CH8 cells and sOR cells stably expressing hepatitis C virus core proteins

PH5CH8 cells and sOR cells were infected with retrovirus vectors encoding different types of HCV core proteins, as described previously (19, 20). At 2 days post-infection, the culture medium was exchanged for a fresh medium containing blasticidin (20 μ g/ml) for PH5CH8 cells or blasticidin (20 μ g/ml) and G418 (0.3 mg/ml) for sOR cells. The culture was continued for 3 weeks so as to select the cells stably expressing the core proteins.

Quantitative reverse transcription-polymerase chain reaction analysis

Total cellular RNA was extracted using an Isogen extraction kit according to the manufacturer's protocol (Nippon Gene, Tokyo, Japan). The quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed by real-time PCR using a Light Cycler (Roche Diagnostics, Mannheim, Germany) as described previously (20–22).

Luciferase reporter assay

For the dual luciferase assay, we used firefly luciferase reporter vectors encoding the 2'-5'-oligoadenylate synthetase (2'5'OAS) promoter, IFN-induced double-stranded RNA-activated protein kinase (PKR) promoter, IFN-stimulated response element (ISRE), and pRL-CMV, which expressed *Renilla* luciferase as described previously (15, 16). Plasmids were transiently transfected into the cells (2.0×10^4 cells/well in 24-well plates) using the FuGene6 transfection reagent (Roche Diagnostics) and cultured for 48 h. The cells were treated with human IFN- α (Sigma, St Louis, MO, USA) and/or RBV at the indicated doses for 6 h before harvest. The RBV was kindly provided by Yamasa (Chiba, Japan) (23). A whole cell lysate was prepared and assayed for firefly and *Renilla* luciferase activities according to the manufacturer's protocol (Promega, Madison, WI, USA). A Lumat LB9507 luminometer (Berthold, Bad Wildbad, Germany) was used to detect luciferase activity. The relative luciferase activity was normalized to the activity of *Renilla* luciferase. The data represent the means of the normalized luciferase activities of triplicate assays. The protocol for the *Renilla* luciferase assay to quantify HCV replicon

RNA was described previously (14). Briefly, 2.0×10^4 cells were plated onto 24-well plates in triplicate and were cultured for 12 h. The cells were treated with human IFN- α and/or RBV at the indicated doses for 48 h, and then harvested with *Renilla* lysis reagent (Promega) and subjected to a luciferase reporter assay according to the manufacturer's protocol. All the luciferase assays were repeated at least three times.

Western blot analysis

Preparations of cell lysates, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed as described previously (15, 16). The antibodies used in this study were those against Core (CP-11; Institute of Immunology, Tokyo, Japan), NS3 (Novocastra Laboratories, Newcastle, UK) and β -actin (AC-15; Sigma). Immuno-complexes were detected using the Renaissance enhanced chemiluminescence assay (PerkinElmer Life Science, Boston, MA, USA).

Cell proliferation analysis

The PH5CH8 cells (5.0×10^3 cells/well) or the sOR cells (2.5×10^3 cells/well) stably expressing HCV core proteins were plated onto 96-well plates, cultured for 24, 48 or 72 h and subjected to the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instruc-

tions (cell proliferation kit I; Roche) as described previously (24).

Results

No changes in the levels of interferon-stimulated genes were detected in response to the substitutions at hepatitis C virus core amino acid 70 and/or amino acid 91 in transiently transfected cells

The constructed plasmids with the four different combinations of HCV core aa70 and aa91 substitutions were transiently transfected into PH5CH8, HepG2, HuH-7 and Li23 cells with lipofection. The levels of 2'5'OAS promoter activities were calculated as the luciferase activities at 48 h after transfection. As shown in Figure 2, the different types of HCV core proteins did not show clear differences in basal levels or enhanced levels with IFN- α stimulation, although the levels of CoreR70L91 transfection were slightly higher than the transfection levels for the other core proteins in PH5CH8 cells. As for PH5CH8 cells, we also evaluated the expression of HCV core proteins with Western blot analysis, and there were no obvious differences as shown in Figure 3. There were no differences either in the levels of the PKR promoter or in ISRE activities (data not shown). These results indicate that the levels of IFN-stimulated genes might not be affected by substitutions of the HCV core proteins at aa70 and/or aa91 in transiently transfected cells.

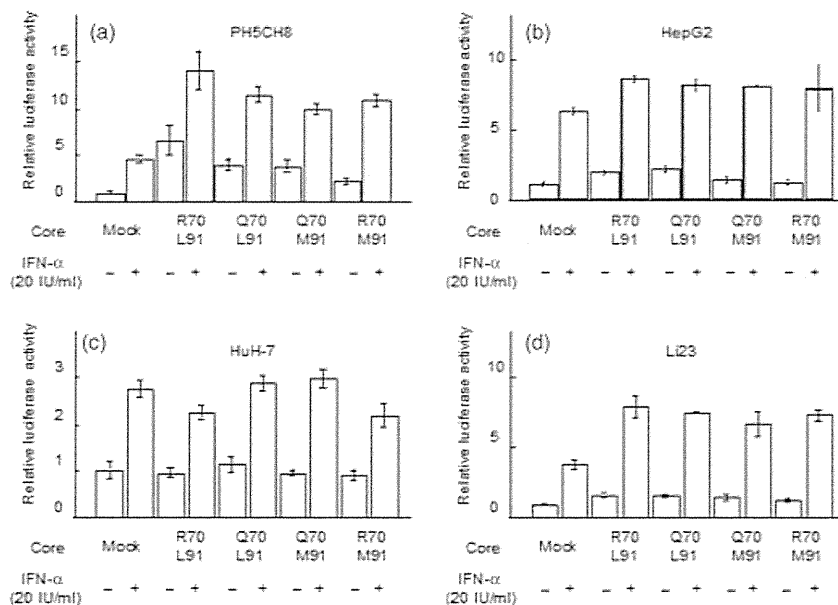


Fig. 2. 2'5'oligoadenylate synthetase (OAS) promoter activity of cells transiently transfected with the hepatitis C virus (HCV) core. The constructed plasmids with the four different types of HCV core aa70 and aa91 were transiently transfected into the cells (2.0×10^4 cells/well in 24-well plates) with lipofection. The cells were treated with IFN- α (20 IU/ml) for 6 h before harvest. The levels of 2'5'OAS promoter activities were calculated as the luciferase activities at 48 h after transfection. The figures show the results using PH5CH8 (a), HepG2 (b), HuH-7 (c) and Li23 cells (d).

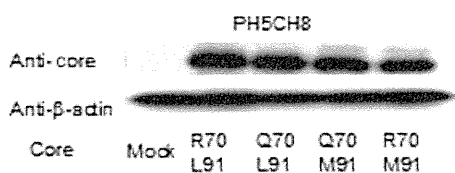


Fig. 3. The expression of hepatitis C virus (HCV) core proteins in the cells transiently transfected with the HCV core. The expressions of HCV core proteins were evaluated with Western blot analysis for PH5CH8. The constructed plasmids with the four different types of HCV core aa70 and aa91 were transiently transfected into the cells (2.0×10^5 cells/well in six-well plates) with lipofection. The cells were collected at 48 h after transfection.

The intracellular antiviral activities were augmented by hepatitis C virus core transduction, although they might not be affected by the amino acid 70 and/or amino acid 91 substitutions in the hepatitis C virus core stably transduced cells

To evaluate the interaction between the stable expression of HCV core proteins and intracellular antiviral activity, we prepared PH5CH8 cells that stably expressed HCV core proteins with retroviral transduction. The 2′/5′OAS mRNA levels were measured with real-time LightCycler PCR and normalized with β -actin mRNA levels. As shown in Figure 4a, there were no clear differences in the basal levels or IFN- α -enhanced levels among the four transduced cell lines and the mock-transduced cells, although the expression levels of HCV core protein stably transduced cells were slightly higher than that of mock-induced cells. Western blot analysis showed that all these cell lines had similar levels of HCV core proteins (data not shown). Next, we monitored the association between the stable expression of HCV core proteins and RNA replication in an HCV replicon system. As we considered that a small difference of antiviral activity might be difficult to detect with real-time PCR, sOR cells were used for the monitoring. These cells are subgenomic HCV-RNA replicating cells with *Renilla* luciferase genes, and the replicon RNA can be quantified as luciferase activity. Measurement of luciferase activity is a useful and accurate means of quantifying the replicon RNA, because its sensitivity is much better than that of real-time PCR (14). We prepared the sOR cells expressing the HCV core proteins with retroviral transduction and stimulated the cells with IFN- α for 48 h. The level of antiviral activity, which was calculated as the median effective concentration (EC_{50}) of IFN- α , demonstrated that the cells transduced with the HCV core proteins showed a better response to IFN- α stimulation, but there was no difference in response among the cells expressing the different types of HCV core proteins, which is consistent with the results for the transient transfection of HCV core proteins (Fig. 4b). Western blot analysis demonstrated that the expressions of the HCV core and NS3 proteins did not show a clear difference among the sOR cells, irrespective of the types of HCV core proteins (Fig. 4c). As shown in Figure 4d, the results of the MTT assay demonstrated that the expressions of different types

of HCV core proteins were not associated with cell proliferation in PH5CH8 cells or sOR cells. These results indicate that the intracellular antiviral activities are augmented in the presence of HCV core proteins, but they are not altered by the different substitutions at HCV core aa70 and/or aa91 in the cells stably expressing the HCV core.

Co-stimulation of interferon- α with ribavirin did not alter the association of hepatitis C virus core proteins with intracellular antiviral activity

Next, we hypothesized that co-stimulation of RBV might modulate the antiviral activity of the cells with different types of HCV core proteins. As shown in Figure 5, we evaluated the antiviral activity by quantifying HCV replicon RNA and 2′/5′OAS promoter activity in the presence of IFN- α and RBV. The dose of RBV (20 μ M) was determined based on the clinically used dose and the cell reactivity to RBV in our previous report (23). The HCV replications were suppressed (Fig. 5a), and the levels of 2′/5′OAS promoter activities were enhanced (Fig. 5b and c) with a smaller dose of IFN- α , compared with IFN- α stimulation alone, indicating that RBV exerted an additive effect. Interestingly, the stimulation with RBV alone did not show any enhancement of 2′/5′OAS promoter activity. These results indicate that the intracellular antiviral activities are augmented by costimulation with IFN- α with RBV, and that they are not altered by changes in the HCV core aa70 and/or aa91 substitutions.

Specific amino acid substitutions of hepatitis C virus core proteins were not detected in response to interferon- α treatment in the genome-length hepatitis C virus-RNA replicating cells

Next, we hypothesized that specific mutations in the HCV core region occur during IFN- α treatment. The two kinds of genome-length HCV-RNA replicating cells with different core aa70 and aa91 substitutions were cultured for 3 weeks with low-dose IFN- α stimulation, and then the core sequences were compared. We used OR6 and AH1 cells, both of which are genome-length HCV-RNA replicating cells (25, 26). OR6 cells have core proteins with glutamine at aa70 and leucine at aa91, corresponding to CoreQ70L91. AH1 cells have core proteins with arginine at aa70 and leucine at aa91, corresponding to CoreR70L91. After 3 weeks of culture with 12.5 IU/ml of IFN- α , several IFN- α - and G418-resistant colonies were obtained. We cultured them further without IFN- α stimulation and spread the colonies individually on 24-well plates. To examine the aa sequences of the core proteins in the colonies derived from OR6 and AH1 cells, RT-PCR was performed for the region encoding the HCV core protein, and the obtained PCR products were cloned into pBlueScript II, as described previously (27). The plasmid inserts of 10 clones each were sequenced. The results revealed that there were no specific changes of the aa sequence of HCV core

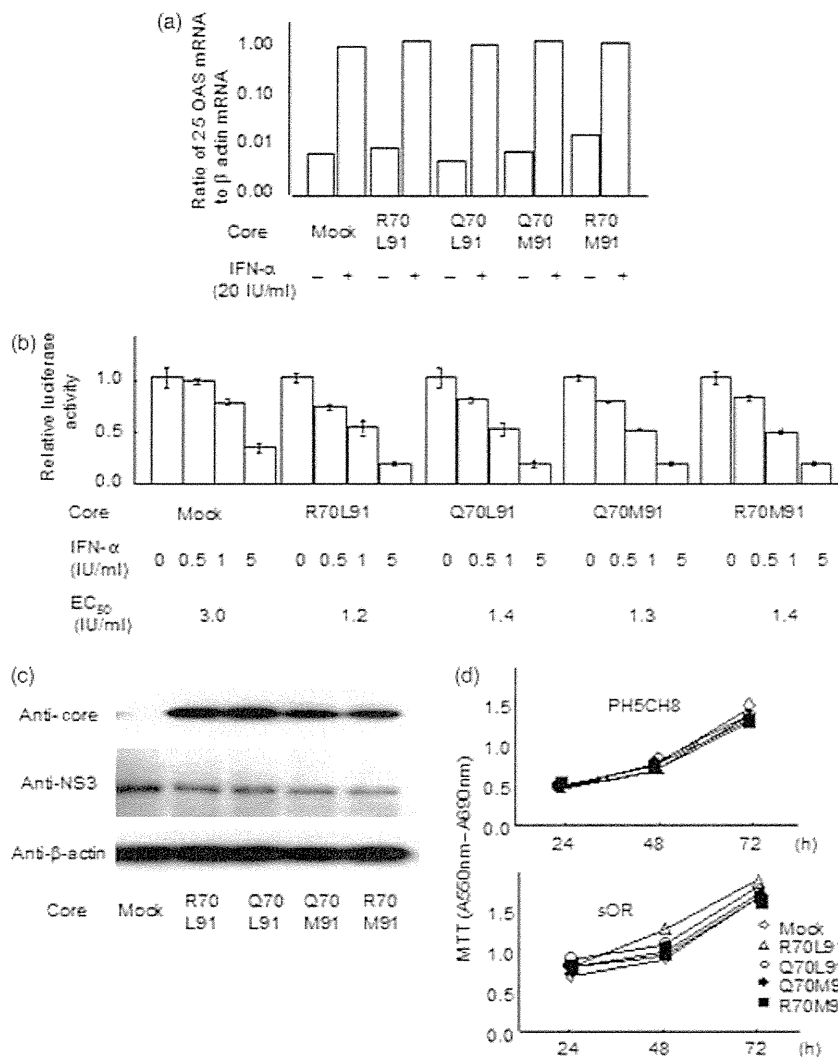


Fig. 4. Antiviral activity of cells stably transduced with hepatitis C virus (HCV) core proteins. The antiviral activity of PH5CH8 cells stably transduced with the HCV core were evaluated by 2'5' oligoadenylate synthetase (OAS) mRNA levels (a). The cells (2.0×10^5 /well in six-well plates) were cultured for 48 h, and treated with IFN- α (20 IU/ml) for 6 h before harvest. 2'5' OAS mRNA levels were measured with real-time LightCycler PCR and normalized with β -actin mRNA levels. The sOR cells stably expressing the different types of HCV core (2.0×10^4 cells/well in 24-well plates) were stimulated with IFN- α for 48 h, and the HCV RNA levels of the cells were calculated as the percent relative *Renilla* luciferase activity [relative RL activity (%)] and compared by evaluating the EC₅₀ of IFN- α (b). sOR cells stably transduced with the HCV core (2.0×10^5 /well in six-well plates) were cultured for 48 h, and prepared for Western blot analysis with anti-core, anti-NS3 or anti- β -actin antibody (c). The PH5CH8 cells (5.0×10^3 cells/well) or the sOR cells (2.5×10^3 cells/well) stably expressing HCV core proteins were plated onto 96-well plates, cultured for 24, 48 or 72 h and subjected to MTT assay. The results for PH5CH8 cells are shown in the upper panel of Figure 4d and those for sOR cells are shown in the lower panel.

proteins from that of the original cells. The OR6 cells retained glutamine at aa70 and leucine at aa91, while the AH1 cells retained arginine at aa70 and leucine at aa91. These results suggest that the aa at positions 70 and 91 are stable during IFN- α treatment.

Discussion

Recent studies on HCV-infected patients have suggested that HCV core proteins with substitutions at aa70 and/or

aa91 may be significantly associated with NVR to IFN- α /RBV therapy, and that patients with aa70 substitutions of arginine to glutamine often have slow or no decrease in HCV-RNA levels during the early phase of IFN- α treatment (6–9). However, the associations between HCV core aa70 and/or aa91 substitutions and the level of antiviral activity have not been determined *in vitro*. We hypothesized that the aa at the HCV core positions 70 and/or 91 would be associated with the intracellular antiviral environment in HCV-infected cells, and

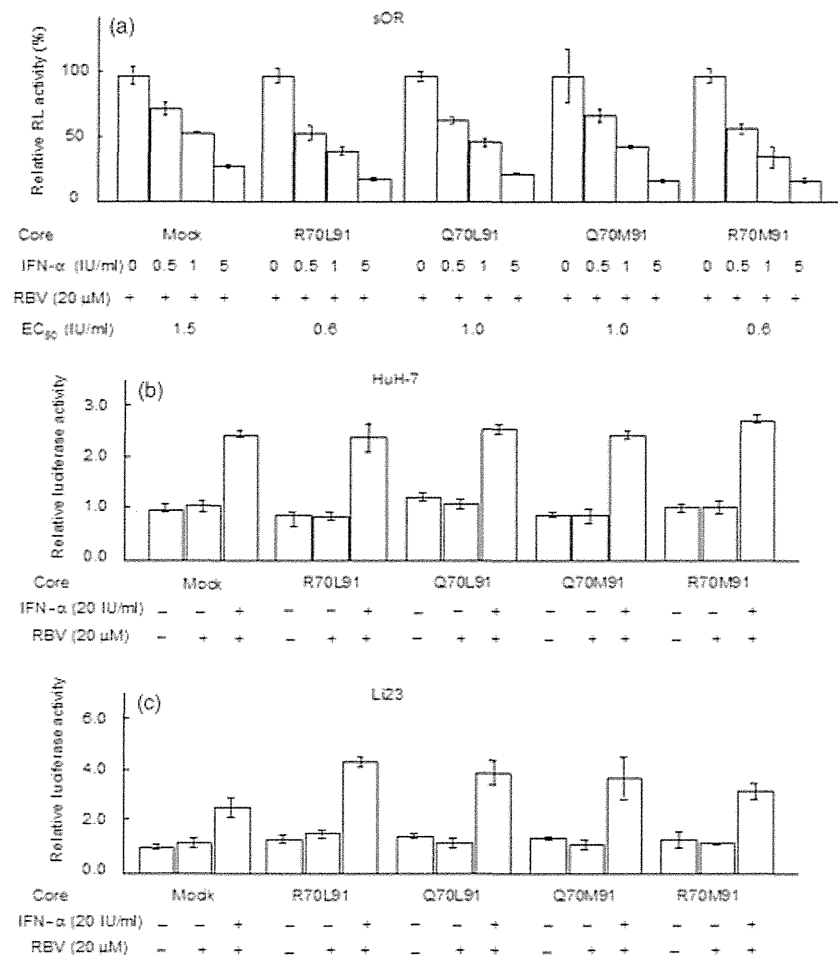


Fig. 5. Antiviral activity with stimulation of interferon (IFN)- α in combination with ribavirin (RBV). The sOR cells (2.0×10^4 cells/well in 24-well plates) stably expressing the different types of HCV core were stimulated with the indicated doses of IFN- α and 20 μ M of RBV for 48 h, and the hepatitis C virus (HCV) RNA levels of the cells were calculated as the percent relative *Renilla* luciferase activity [relative RL activity (%)]. The EC₅₀ of IFN- α was calculated as shown in (a). HuH-7 cells and Li23 cells were seeded (2.0×10^4 cells/well in 24-well plates), and the different types of HCV core were transiently transfected into the cells with lipofection. The transfected cells were treated with 20 IU/ml of IFN- α and 20 μ M of RBV for 6 h before harvest. The levels of 2'5'oligoadenylate synthetase promoter activities were calculated as the luciferase activities after 48 h of transfection. The figures show the results using HuH-7 cells (b) and Li23 cells (c).

evaluated the differences in IFN- α -induced antiviral activities according to the aa at these positions. Our results suggest that differences in the aa at HCV core positions 70 and/or 91 are not associated with the intracellular antiviral activity in HCV-infected cells.

The HCV core protein has been reported to exert an effect on a variety of cellular functions, including apoptosis, RNA metabolic processes, inflammation, cholesterol metabolism and protein catabolism (1, 28–32), and is currently considered to play important roles in persistent infection. In terms of a direct interaction between the HCV core protein and antiviral activity, Naganuma *et al.* (15) reported that 2'5'OAS promoter activity was activated in PH5CH8 cells when the cells were transiently transfected with the HCV core protein, and their deletion mutant analysis indicated that HCV core aa70 and/or

aa91 substitutions were not associated with activated 2'5'OAS promoter activity, which is consistent with our present results that the antiviral activities were not associated with the aa at the core position 70 or 91 in the transiently transfected cells. Furthermore, we evaluated the antiviral activity in cells stably transduced with the HCV core protein by precisely measuring the levels of HCV replicon RNA based on luciferase activity. For this purpose, we used sOR cells, which are subgenomic HCV-RNA-replicating cells. The sOR cells facilitates the monitoring of HCV replication, although it lacks the steps of budding or HCV re-infection to other cells. Future studies will be required to assess these steps according to the different substitutions at HCV core aa70 and/or aa91, and infectious HCV production systems from the HCV genotype 1b strain will be required for this purpose.

It is not clear whether the aa at the core positions 70 or 91 can be changed through IFN- α therapy or disease progression. Our results revealed that the aa at the core positions 70 or 91 in the cells with monoclonal HCV replication were not substituted after 3 weeks of IFN- α treatment. The substitution of arginine to glutamine at aa70, or of leucine to methionine at aa91 might not occur in the infected cells, but rather through a change in the dominant virus, such as through resistance to IFN- α therapy or during disease progression.

In conclusion, the antiviral activities in response to IFN- α or IFN- α /RBV treatment were augmented by HCV core transduction. However, the levels of these activities were not associated with changes in the aa at HCV core positions 70 or 91 by *in vitro* analysis with immortalized hepatocytes or HCV-RNA replicating cells.

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Hydroxyurea as an inhibitor of hepatitis C virus RNA replication

Akito Nozaki · Manabu Morimoto · Masaaki Kondo · Takashi Oshima · Kazushi Numata · Shin Fujisawa · Takeshi Kaneko · Eiji Miyajima · Satoshi Morita · Kyoko Mori · Masanori Ikeda · Nobuyuki Kato · Katsuaki Tanaka

Received: 13 October 2009 / Accepted: 29 December 2009 / Published online: 5 March 2010
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Abstract Hepatitis C virus (HCV) is the main causative agent of chronic liver disease, which may develop into liver cirrhosis and hepatocellular carcinoma. By using a recently developed reporter assay system in which genome-length HCV RNA replicates efficiently, we found that hydroxyurea (HU), a DNA synthesis inhibitor, inhibited HCV RNA replication. Moreover, we demonstrated that the anti-HCV activity of the combination of IFN- α and HU was higher than that of IFN- α alone. These results suggest that HU may be an effective anti-HCV reagent that can be used not only singly but also in combination with IFN- α to treat chronic hepatitis C.

Introduction

Hepatitis C virus (HCV) is the main causative agent of chronic hepatitis C (CHC), which can develop into liver cirrhosis and hepatocellular carcinoma [4, 12, 19]. HCV infection is a global health problem, with over 170 million people being infected with the virus [24]. HCV genotype 1 is the major genotype found in Japan, the United States, and many other countries. Unfortunately, less than 50% of the patients infected with HCV of this genotype respond to the standard combination therapy of pegylated interferon (IFN) and ribavirin [6, 15]. In order to develop a more effective therapy especially for these patients, we recently developed a genome-length HCV RNA (strain O of genotype 1b) replication reporter system (OR6), which has been an effective screening tool [9, 17]. By using this

A. Nozaki (✉) · M. Morimoto · M. Kondo · T. Oshima · K. Numata · K. Tanaka
Gastroenterological Center, Yokohama City University Medical Center, 4-57, Urafune-cho, Minami-ku, Yokohama 232-0024, Japan
e-mail: akino@yokohama-cu.ac.jp

A. Nozaki
Department of Transfusion Medicine, Yokohama City University Medical Center, 4-57, Urafune-cho, Minami-ku, Yokohama 232-0024, Japan

S. Fujisawa
Department of Hematology, Yokohama City University Medical Center, 4-57, Urafune-cho, Minami-ku, Yokohama 232-0024, Japan

T. Kaneko
Respiratory Disease Center, Yokohama City University Medical Center, 4-57, Urafune-cho, Minami-ku, Yokohama 232-0024, Japan

E. Miyajima
Department of Laboratory Medicine and Clinical Investigation, Yokohama City University Medical Center, 4-57, Urafune-cho, Minami-ku, Yokohama 232-0024, Japan

S. Morita
Department of Biostatistics and Epidemiology, Yokohama City University Medical Center, 4-57, Urafune-cho, Minami-ku, Yokohama 232-0024, Japan

K. Mori · M. Ikeda · N. Kato
Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8558, Japan

system, we found that statins inhibited HCV RNA replication [10, 11]. Moreover, Bader et al. [2] conducted a clinical study on the basis of our previous findings and reported that fluvastatin also exhibited anti-HCV activity in CHC patients. In this study, we used this system to screen a series of drugs that have not yet been used against HCV. We particularly focused on the compounds that have been found to be effective in the case of other viruses. Of these compounds, we found that hydroxyurea (HU), a DNA synthesis inhibitor, inhibited HCV RNA replication.

We used OR6 cells to evaluate whether HU alone could inhibit the replication of genome-length HCV RNA because these cells are considered to constitute a reliable

system for monitoring HCV RNA replication. The OR6 cell line was cloned from HuH-7 cells with replicating ORN/C-5B/KE (strain O of genotype 1b) RNA (Fig. 1a), as described previously [9]. We used cyclosporin A (CsA), a cyclophilin inhibitor, as the positive control, as reported previously [25, 26]. In order to monitor the antiviral effect of HU, IFN-alpha, and CsA, OR6 cells were plated onto 24-well plates (1.5×10^4 cells per well) and cultured for 24 h. Subsequently, the cells were treated with several concentrations of human IFN-alpha, HU, and CsA or their combination for 72 h. After treatment, luciferase activity was measured using the *Renilla* luciferase (RL) assay system (Promega, USA) as described previously [9].

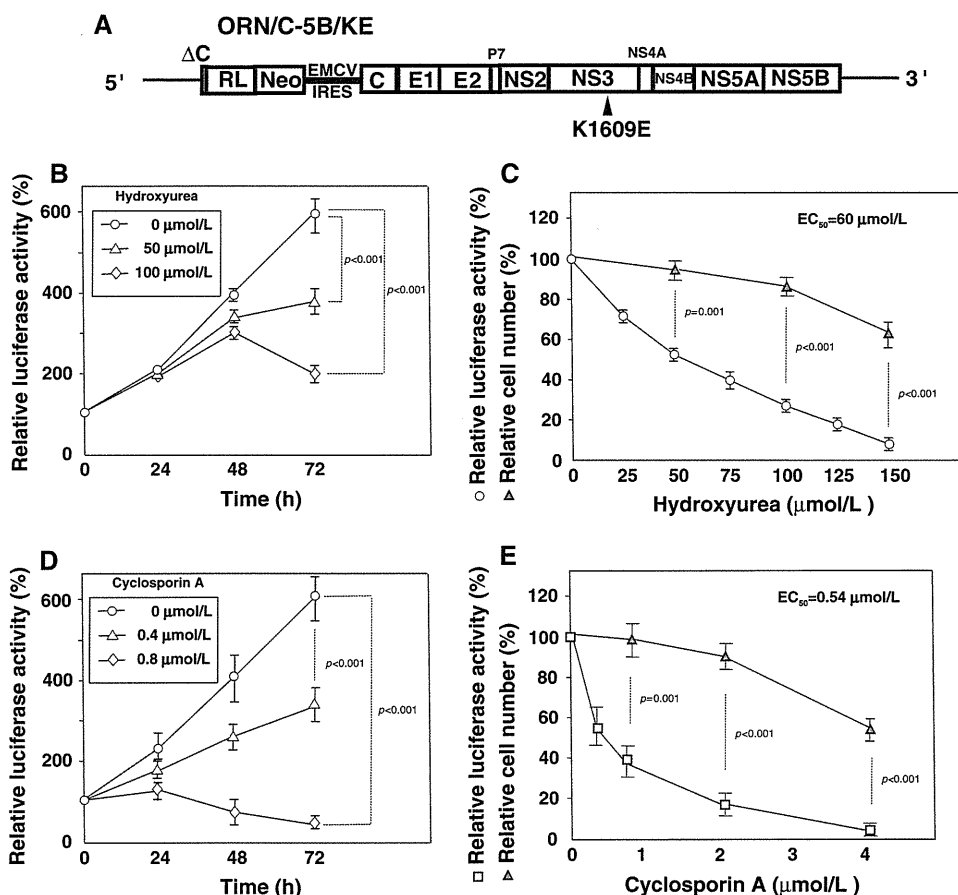


Fig. 1 **a** Schematic representation of the gene organization during replication of genome-length hepatitis C virus (HCV) RNA in OR6 cells. The position of the adaptive mutation K1609E is indicated by a black triangle **b** Hydroxyurea (HU) alone inhibits HCV RNA replication in OR6 cells. OR6 cells were treated with HU (0, 50, and 100 $\mu\text{mol/L}$) for 24, 48, or 72 h. Subsequently, the *Renilla* luciferase (RL) assay was performed. Luciferase activities were compared statistically using Student's *t* test, and the values are represented as the mean [standard deviation (SD)] ($n = 3$). **c** Dose-response curve of HU. The EC_{50} of HU was found to be approximately 60 $\mu\text{mol/L}$. Cell viability after HU treatment was also determined. OR6 cells were cultured for 72 h in the presence or absence of HU (50, 100, and 150 $\mu\text{mol/L}$), and the number of viable

cells was determined. The relative number of viable cells (%) determined for each concentration is presented. The values were compared statistically using Student's *t* test and are represented as the mean (SD) ($n = 3$). **d** Cyclosporin A (CsA) alone inhibited HCV RNA replication in OR6 cells. OR6 cells were treated with CsA (0, 50, and 100 $\mu\text{mol/L}$) for 24, 48, or 72 h. Subsequently, the RL assay was performed. Luciferase activities were compared statistically using Student's *t* test, and the values are represented as the mean (SD) ($n = 3$). **e** Dose-response curve of CsA. The EC_{50} of CsA was calculated to be approximately 0.54 $\mu\text{mol/L}$. The relative number of viable cells (%) determined for each concentration is presented. The values were compared statistically using Student's *t* test and are represented as the mean (SD) ($n = 3$).

Luciferase activity was detected using a manual Monolight 3010 luminometer (Becton-Dickinson, USA). HU, which was over 98% pure, human IFN- α , and CsA were purchased from Sigma-Aldrich, USA.

In this study, we found that HU alone inhibited the replication of genome-length HCV RNA in OR6 cells (Fig. 1b). According to the dose–response curve obtained after 72 h treatment with HU (up to 150 $\mu\text{mol/L}$), the median effective concentration (EC_{50}) of HU was calculated as 60 $\mu\text{mol/L}$ (Fig. 1c).

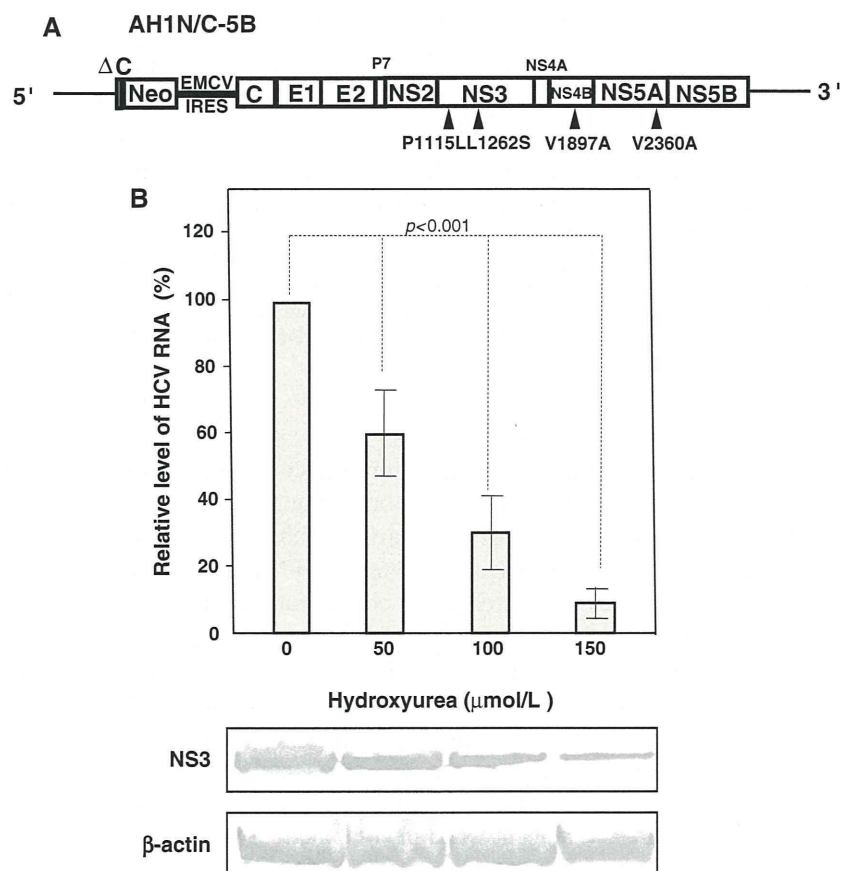
Additionally, it has been reported that the proliferation of the HCV replicon is dependent on host cell growth [18]. However, it remained unclear whether the inhibitory effects of HU on HCV RNA replication are attributable to its cytotoxic effects. In order to examine this possibility, we assessed the cytotoxic effects of HU on OR6 cells. The cells were treated without HU or with HU at final concentrations of 50, 100, and 150 $\mu\text{mol/L}$ for 72 h in the absence of G418. After staining with trypan blue dye (Invitrogen, USA), the number of viable cells was determined using an improved Neubauer hemocytometer. Moderate cytotoxic effects were observed at 150 $\mu\text{mol/L}$ HU but not at concentrations below 100 $\mu\text{mol/L}$ (Fig. 1c). The same experiment was performed for CsA (Fig. 1d).

The EC_{50} of CsA was 0.54 $\mu\text{mol/L}$, and the cytotoxic effect of CsA was found to be similar (Fig. 1e).

Next, we used a reporter assay in which cured OR6 (OR6c) cells were transfected with plasmids encoding RL driven by the internal ribosome entry site (IRES) of encephalomyocarditis virus (EMCV) (pEMCV-RL). The cells were then treated with HU as described previously [10]. This assay revealed that HU did not inhibit RL activity and the IRES of EMCV, suggesting that HU can independently inhibit HCV RNA replication (data not shown).

We previously reported a novel RNA replication system comprising a hepatitis C virus strain (AH1) derived from a patient with acute hepatitis C [16]. In order to exclude the possibility that the anti-HCV activity is overestimated by using the OR6 system, we used AH1 cells as the second HCV replicon system. Consequently, a single cloned AH1 cell line that supported the efficient replication of genome-length HCV RNA was obtained from the OR6c cells (Fig. 2a). We performed both RT-qPCR and Western blot analysis using AH1 cells as reported previously [16]. HU inhibited not only HCV RNA replication but also NS3 synthesis in a dose-dependent manner (Fig. 2b).

Fig. 2 Anti-HCV activity of HU in AH1 cells showing HCV RNA replication. **a** Schematic representation of the gene organization of the genome-length HCV RNA in AH1 cells. The positions of the adaptive mutations P1115L, L1262S, V1897A, and V2360A are indicated by black triangles. **b** AH1 cells were treated with HU for 72 h. The extracted total RNAs and cell lysates were subjected to real-time quantitative polymerase chain reaction (RT-qPCR) and Western blot analysis for the detection of the HCV 5'-untranslated region (UTR) (*upper panel*) and non-structural (NS3) protein (*lower panel*), respectively. Relative HCV RNA levels were compared statistically using a linear regression analysis, and the values are represented as the mean (SD) ($n = 3$)



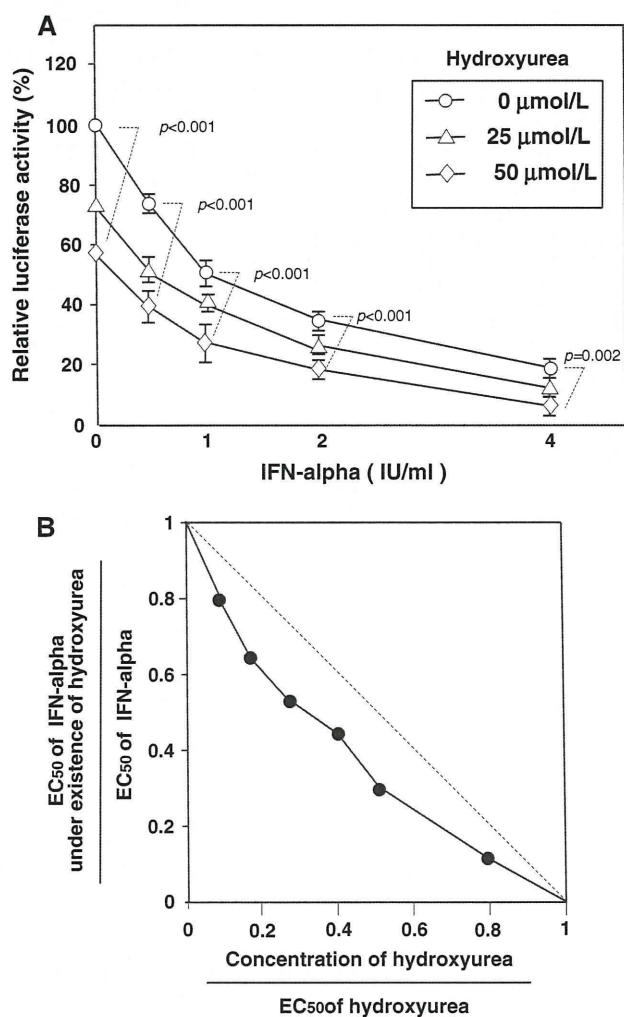


Fig. 3 HU enhances the inhibitory effect of interferon (IFN)-alpha on HCV RNA replication. **a** OR6 cells were cotreated with IFN-alpha (0, 0.5, 1, 2, and 4 IU/mL) and HU (0, 25, and 50 µmol/L) for 72 h, after which the RL assay was performed. Luciferase activities were compared statistically using linear regression analysis, and the values are represented as the mean (SD) ($n = 3$). **b** An isobologram showing the synergistic inhibitory effect of HU and IFN-alpha on HCV RNA replication

Furthermore, we examined the inhibitory effects of the IFN-alpha and HU combination on genome-length HCV RNA replication. The EC_{50} of IFN-alpha was found to be 1.2 IU/mL (Fig. 3a). Dose–response curves of IFN-alpha were obtained for each of the following concentrations of HU: 0, 25, and 50 µmol/L. The curves shifted to the left with increasing concentrations of HU (Fig. 3a), indicating that the co-treatment was more effective than treatment with IFN-alpha alone. Isobologram analysis revealed a synergistic anti-HCV activity of the IFN-alpha and HU combination (Fig. 3b). These results suggest that HU can be used as an anti-HCV reagent when in combination with IFN-alpha.

In this study, we found that HU, a DNA synthesis inhibitor, inhibited genome-length HCV RNA replication. The EC_{50} of HU determined in this study (60 µmol/L) was lower than that of ribavirin ($EC_{50} = 76\text{--}26$ µmol/L), which was determined in previous studies [17, 22]. Moreover, the maximal tolerated dose (MTD) of HU in humans was estimated as 800 mg/m² when administered orally every 4 h, and the mean plasma peak concentration increased to 2,480 µmol/L [3]. This is substantially higher than the EC_{50} determined in this study. Thus, HU may be used alone for clinical applications. Furthermore, we demonstrated that the combination of IFN-alpha and HU exhibited greater anti-HCV activity than IFN-alpha alone. HU has been successfully combined with IFN-alpha to improve the efficiency of IFN therapy for leukemia [8, 23]; hence, HU is considered to be of potential use in combination with IFN-alpha.

Although HU is primarily used in patients with chronic leukemia, melanoma, and other solid tumors [1], the activity of HU against viruses such as human immunodeficiency virus (HIV) and herpes simplex virus (HSV) has been reported recently [13, 20]. Several clinical trials of HU in combination with a protease inhibitor have been performed on HIV-infected patients [14]. These reports state that the underlying mechanism may involve the inhibition of viral DNA synthesis. Moreover, another mechanism, prevention of G1/S transition by HU, may play an important role in HIV inhibition [5, 7]. As shown in Fig. 1b, HU showed antiviral activity 48–72 h after administration. These findings indicate that the mechanism underlying the direct inhibitory effect of HU involves an effect of HU on the cell cycle. Recently, cyclophilin B (CypB), a host factor, was identified as a critical factor for efficient HCV genome replication [26]; other non-identified host factors could also be crucial for efficient replication. Therefore, HU may inhibit HCV RNA replication by interacting with these important host factors. Drugs that target such host cell factors instead of the virus itself are less likely to induce drug resistance. Another possible mechanism involves the enhancement of the antiviral activity of IFN via the upregulation of the IFN receptor. Tamura et al. [21] reported that the expression of IFN receptor was upregulated in HU-treated leukemia cell lines; however, HU did not upregulate the expression of IFN regulatory factor (IRF) in the absence of IFN-alpha. Both mechanisms are necessary to explain the synergistic anti-HCV activity of the IFN-alpha and HU combination. Further analysis is required to clarify these points. On the other hand, both HU and IFN-alpha have hematological side effects in the clinical setting. Therefore, careful examination is necessary before combination therapy with these two compounds can be initiated.