in the Frac 6 HCV particle. Indeed, densitometric analysis of the blot indicated that the level of both E1 and E2 proteins in Frac 8 was 1.5-fold higher than that in Frac 6. This higher level of E1 and E2 proteins in Frac 8 may be the reason why the Frac 8 HCV particles had higher infectivity. Interestingly, the expression level of the core protein relative to that of the envelope proteins was different in infected cells and in purified HCV particles (Figure 3). These data suggest that only a limited amount of the envelope protein was incorporated into HCV particles.

We compared the potential of the purified HCV particles to function as a vaccine with that of HCV-envelope components. For this purpose, recombinant HCV E1 and E2 proteins were prepared and different doses of recombinant E2 (rE2), or recombinant E1 (rE1) plus rE2, were injected into mice with adjuvant. Other groups of mice were immunized with the HCV-particle vaccine (Frac 6 or Frac 8) using a dose of 2 pmol of HCV core per mouse. Because it was calculated that HCV particles that included 2 pmol of HCV core protein contained approximately 20 ng of recombinant E1 and E2, we used 20–2000 ng of rE2 or 20 or 200 ng each of rE1 plus rE2 proteins for comparative purposes. Although the sera from all immunized mice contained anti-E2 antibodies, and immunization with 200 or 2000 ng of recombinant E2 protein induced antibody production with the highest efficiency, HCV-particle immunization displayed the highest virus-neutralizing effect. Based on these results, it was concluded that purified HCV particles may be a more potent prophylactic HCV vaccine candidate than a vaccine that is composed of recombinant viral components. There are several possible explanations for the observed differences between these 2 types of vaccines. First, HCV envelope proteins that are a part of HCV particles are properly folded, which would maintain their function, whereas the recombinant envelope proteins may not be properly folded, and thus may lose their antigenicity. Second, other factors associated with the HCV particles may be important for inducing neutralizing antibodies. To address these possibilities, we adsorbed the anti-envelope antibodies in iHCV-IgG with recombinant envelope proteins. This procedure reduced, but did not completely abolish, the neutralizing effect of iHCV-IgG toward HCVcc infection. It is possible that if the recombinant envelope proteins were not folded properly as mentioned earlier, then not all of the anti-envelope antibodies were removed in this adsorption process. However, this possibility is considered unlikely because virus-neutralizing antibodies were induced by both immunization with the HCV particles and with the recombinant envelope proteins. This result suggests that HCV particles may include other factors, such as lipoproteins, that are important for HCV infection.[15–17] It also is necessary to compare the immunogenic activity of the inactivated virus vaccine with the potential immunogenic activities of recombinant HCV-like particles and E1-E2 heterodimers. Furthermore, attention needs to be paid as to whether the inactivated purified vaccine may induce autoantibodies against human proteins associated with the viral particles.

To confirm the in vivo efficacy of HCV-particle–induced neutralizing antibodies, the ability of iHCV-IgG to protect infected animals against HCV challenge was examined using human liver chimeric uPA-SCID mice. Interestingly, iHCV-IgG could protect against HCV challenge, at least at the lower virus challenge dose, although the mice challenged with the higher virus doses became infected with HCV. This result indicated that the HCV-particle vaccine could exert a prophylactic anti-HCV effect in vivo by induction of virus-neutralizing antibodies. However, the therapeutic effect of this vaccine is not yet clear and an understanding of its effect will require detailed study of its effect on cellular immune responses such as the cytotoxic T lymphocyte response. In addition, the prophylactic effect was observed only partially in the in vivo experiment. The reason for this insufficient protection is unclear; however, induction with a higher titer of neutralizing antibody may be needed, using adjuvants or more effective forms of the vaccine such as recombinant HCV-like particles. It also may be important to induce protective cellular immune responses in addition to humoral immune responses; effective cytotoxic T-lymphocyte responses may be necessary for a prophylactic effect against infection at higher virus doses.

Prophylactic and therapeutic HCV vaccines have been developed previously and some of these vaccines have been tested in clinical trials.[5,18,19] An HCV vaccine should be able to induce humoral and cellular immune response with specific anti-HCV effects. Previous vaccine candidates that have been tested have included recombinant proteins,[20–24] peptides,[25–29] DNA,[30] and viral vectors[31,32] with or without appropriate adjuvants. Recombinant E1 and E2 proteins with an MF59 adjuvant were developed by Chiron/Novartis (Basel, Switzerland) as a prophylactic vaccine, and the immunogenicity of this vaccine was confirmed in mice and guinea pigs.[33] The recombinant protein vaccine was well tolerated in a phase I clinical study, and induction of anti-envelope antibodies and an envelope-specific T-cell response was observed.[24] In another study, Fab fragments of anti-E2 antibodies that were obtained using a phage display library generated from an HCV-infected donor showed a broad HCV-neutralizing effect in human liver chimeric uPA-SCID mice.[34] A separate study showed that retroviral pseudotype-derived HCV-like particles (HCVLPs) also induced broad neutralizing antibodies in mice and macaques.[35] This result indicated that a prophylactic vaccine may be achievable by activation of the humoral immune response. Moreover, it was reported that insect cell-derived virus-like particles containing HCV structural proteins induced a humoral immune response[36] and stimulated dendritic cells[37] or a cytotoxic T-lymphocyte response.[38] Thus, it is possible that an HCVcc-derived HCV-particle vaccine also has the capacity to function as a therapeutic vaccine.

The HCV vaccine described in this study could be used for protection against HCV infection in high-risk patient groups. Given that the HCV particles are used as the vaccine, it will be necessary to improve the production and

purification of these particles before they can be used. We recently established a serum-free HCV culture system for efficient production and purification of virus particles that may be useful for this purpose.[39] It also will be necessary to examine other cell types for the production of HCV particles because the Huh7 cells used in this study are cells of a hepatoma cell line. Once HCV particle production using an industrial method is established and its safety is evaluated and confirmed, then it is expected that a purified HCV-particle vaccine will be of prophylactic use. Alternatively, a novel vaccine composed of HCV components should be developed using recombinant E1 and E2 proteins that can induce viral neutralizing antibodies with similar or even higher efficiency as HCV particles.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2013.05.007.

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Reprint requests

Address requests for reprints to: Takaji Wakita, MD, PhD, Department of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. e-mail: wakita@nih.go.jp; fax: (81) 3-5285-1161.

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Materials and Methods

RNA Synthesis

RNA synthesis was performed as described previously.[1] Briefly, the pJ6/JFH1 plasmid was digested with *Xba* I and then treated with Mung Bean Nuclease (New England Biolabs, Beverly, MA). The digested plasmid DNA fragment then was purified and used as a template for RNA synthesis. HCV RNA was synthesized in vitro using a MEGAscript T7 kit (Ambion, Austin, TX). The synthesized RNA was treated with DNase I, followed by acid phenol extraction to remove any remaining template DNA.

RNA Transfection

RNA transfection was performed as described previously.[1] Briefly, trypsinized Huh7 cells were washed with Opti-MEM I reduced-serum medium (Invitrogen, Carlsberg, CA) and were resuspended at a density of 7.5×10^6 cells/mL in Cytomix buffer.[2] RNA (10 μg) that was synthesized from pJ6/JFH1 was mixed with 400 μL of the cell suspension and the mixture then was transferred into an electroporation cuvette (Precision Universal Cuvettes; Thermo Hybrid, Middlesex, UK). The cells then were pulsed at 260 V and 950 μF using the Gene Pulser II apparatus (Bio-Rad, Hercules, CA). Transfected cells were transferred immediately to a 10-cm dish containing 8 mL of culture medium. Cell culture supernatants were collected 72 hours after transfection and were passed through a 0.45-μm filter. The filtered culture media then was pooled and concentrated using an Amicon Ultra-15 (100,000 molecular weight cut-off; Millipore, Billerica, MA). The viral infectious titer was determined by immunofluorescence detection of infected foci using naive Huh7.5.1 cells, as described later.

HCV Particle Purification

Huh7 cells were infected with J6/JFH-1 chimeric HCVcc (multiplicity of infection, 0.2). After the infected cells were passaged into CellSTACK (Corning, Corning, NY) and cultured in 2% fetal bovine serum–supplemented medium, the cell supernatant was collected and passed through a 0.45-μm filter. The supernatant was concentrated by ultrafiltration using a hollow fiber UFP-500-C-3MA (GE Healthcare, Little Chalfont, UK) and then was diafiltered with 5 times the volume of phosphate-buffered saline. The concentrated and diafiltered cell supernatants were layered on top of a preformed continuous 10%–60% sucrose gradient or a 20%/60% sucrose cushion in 10 mmol/L Tris, 150 mmol/L NaCl, 0.1 mmol/L EDTA. The gradients were centrifuged using an SW28 rotor (Beckman Coulter, Fullerton, CA) at 28,000 rpm (141,000 $\times$ *g*) for 4 hours at 4°C, and 1-mL fractions were collected from the bottom of the tube. The density of each fraction was estimated by weighing a 100-μL decrease from fractions of a gradient run. The HCV core protein and HCV RNA in each fraction were quantified as described later. Purified HCV was prepared by collecting the peaks of HCV core and HCV RNA, which then were concentrated using an Amicon Ultra-15 (100,000 molecular weight cut-off; Millipore).

Quantification of HCV Core Protein and RNA

The concentration of HCV core proteins in sample aliquots was measured using the HCV Core enzyme-linked immunosorbent assay kit (Ortho Clinical Diagnostics, Tokyo, Japan). Viral RNA was isolated from harvested culture media or from sucrose density gradient fractions using the QiaAmp Viral RNA Extraction Kit (Qiagen, Tokyo, Japan). Copy numbers of HCV RNA were determined by RTD-PCR using an ABI Prism 7500 fast sequence detector system (Applied Biosystems, Tokyo, Japan).[3]

HCV Pseudo-Particle Production

Murine leukemia virus pseudotypes were generated according to methods described previously.[4] Briefly, DNA corresponding to the Gag-Pol packaging construct (3.1 μg), the transfer vector construct (3.1 μg), and the HCV glycoprotein-expressing construct (1 μg) was transfected into 2.5×10^6 293T cells, which had been seeded the day before in 10-cm dishes using the FuGENE6 transfection reagent (Roche Diagnostics, Tokyo, Japan). For construction of a vector that expresses the HCV glycoprotein, the coding region corresponding to aa 132-746 (H77 strain), 132-747 (TH strain), or 132-750 (J6CF strain) was cloned into the pcDNA3.1 vector (Invitrogen). For the negative control, all constructs except the glycoprotein-expressing construct were transfected. The medium (8 mL/dish) was replaced 6 hours after transfection. Supernatants containing the pseudotypes were collected 48 hours later, and were passed through a 0.45-μm filter. The supernatants were stored at -80°C until use.

Preparation of Recombinant Proteins

Each E1 (aa 192-353) and E2 (aa 384-720) coding region of the J6CF strain was cloned into the p3×FLAG-CMV-13 (Sigma, St Louis, MO) vector. Each expression vector then was transfected into COS-1 cells using the Diethylaminoethanol (DEAE)-dextran method. The culture supernatant of the transfected cells was collected and FLAG-tagged E1 (J6E1/FLAG) and FLAG-tagged E2 (J6E2/FLAG) proteins were purified using an anti-FLAG M2-agarose (Sigma) column under native conditions. The buffer containing the purified proteins was changed and the proteins were concentrated by ultrafiltration using Amicon Ultra-15 (Millipore).

Immunization of Mice With the HCV Vaccine

All animal procedures were approved by the Committees on Biosafety and Animal Handling Regulations of the National Institute of Infectious Diseases (Japan). Animal research was performed in compliance with the "Fundamental guidelines for proper conduct of animal experiment and related activities in institutions under jurisdiction (June 2006)" issued by the Ministry of Health, Labour and Welfare. Our animal work also adhered to the principles stated in the guidelines. BALB/c mice (4 weeks old, female) were purchased from SLC Japan (Shizuoka, Japan). Mice were tamed for 1 week before the start of experiments. HCV particles in virus-containing fluid were inactivated with ultraviolet irradiation and were conjugated with the same volume

of a solution containing monophosphoryl lipid A and trehalose-6,6-dimycolate using the Sigma Adjuvant System (Sigma). The conjugate (0.2 mL) was injected intraperitoneally into 5-week-old female BALB/c mice, and blood samples were collected. The same volume of saline was injected as a control. Mice were injected at weeks 0, 2, 4, and 6, and blood was collected at weeks 1, 3, 5, and 7 after injection. Blood samples were collected in a BD Vacutainer SST II (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 1200 × *g* for 10 minutes at room temperature. The supernatants were collected as sera and were heat-inactivated at 56°C for 1 hour. The heat-inactivated sera was used in viral inhibition assay experiments and for IgG purification.

HCV Inhibition Assay

Inhibition of HCV infection of cultured cells was assayed using HCVpp and HCVcc for infection. Naive Huh7.5.1 cells (2×10^4) were seeded into a 48-well plate. HCVpp or HCVcc was mixed with serum that was collected from vaccine-immunized mice and the mixtures then were incubated for 30 minutes at room temperature. Naive Huh7.5.1 cells were inoculated with the virus-antibody mixtures, and after 3 hours the mixtures were removed and the cells were washed once with phosphate-buffered saline. DMEM-10 was added to each well, and the cells were cultured for 72 hours. For the HCVpp assay, cells were washed once with phosphate-buffered saline and lysed with 40 μL/well of Cell Culture Lysis Reagent (Promega, Madison, WI), and luciferase activity was quantified using a Luciferase Assay System (Promega) as described previously.[5] For the HCVcc assay, cells were washed once with phosphate-buffered saline and lysed with 100 μL/well of Passive Lysis Buffer (Promega), and HCV core protein content was quantified using the HCV core enzyme-linked immunosorbent assay kit (Ortho Clinical Diagnostics). Assays were performed in triplicate and infectivity was calculated from the value of the luciferase activity or the HCV core content.

Enzyme Immunoassay

Recombinant J6E1/FLAG or J6E2/FLAG protein (50 ng) was coated onto a 96-well plate (Nunc, Roskilde, Denmark) at 4°C overnight. Each well was blocked with Blocking One (Nacalai Tesque, Kyoto, Japan) for 1 hour at room temperature, and then was washed with phosphate-buffered saline. Sera from immunized mice were diluted 1000-fold in PBS, added to each well, and the plate was incubated for 1-2 hours at room temperature. The sera was discarded and each well was washed twice with phosphate-buffered saline. Horseradish-peroxidase-conjugated anti-mouse IgG antibody (GE Healthcare) was diluted 3000-fold with Blocking One, added into each well, and the plate then was incubated for 1 hour at room temperature. Nonbound antibody was discarded, and peroxidase activity of the horseradish-peroxidase-bound antibody was detected using a peroxidase detection kit (Sumitomo Bakelite, Tokyo, Japan).

HCV Inhibition Assay

Inhibition of HCV infection of cultured cells was assayed using HCVpp and HCVcc for infection. HCVpp or HCVcc was mixed with serum or immunoglobulin that was collected from vaccine-immunized mice and the mixtures then were incubated for 30 minutes at room temperature. Naive Huh7.5.1 cells were inoculated with the virus-antibody mixtures, and after 3 hours the mixtures were removed and the cells were washed once with phosphate-buffered saline. DMEM-10 was added to each well, and the cells were cultured for 72 hours. The cells were lysed and the lysates were assayed as described later.

Western Blot Analysis

The naive Huh7 cells that were used as a negative control and the J6/JFH-1-infected Huh7 cells that were used as a positive control were lysed using Passive Lysis Buffer (Promega), and then centrifuged to remove debris. Protein concentration was quantified using the BCA Protein Assay Kit (Pierce, Rockford, IL). The HCV core of purified HCV particles (0.5 pmol each of Frac 6 and Frac 8) and cell lysate samples (5 μg protein) then were denatured by boiling, resolved on 12% sodium dodecyl sulfate-polyacrylamide gels, and transferred to an Immobilon-P membrane (Millipore, Billerica, MA). Recombinant FLAG-tagged E1 (J6E1/FLAG) and FLAG-tagged E2 (J6E2/FLAG) proteins also were analyzed using 1, 3, 10, and 30 ng protein/lane. The membrane was blocked with BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan), and probed with primary antibodies against HCV core (2H9, 3 μg/mL), E1 (B7567[6], 10 μg/mL), and E2 (AP33, 3 μg/mL; a generous gift from Genentech, Inc, South San Francisco, CA) proteins in Tris-buffered saline containing Tween-20 (20 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 0.1% [vol/vol] Tween-20 [polyoxyethylene sorbitan monolaurate]) containing 10% (vol/vol) BlockAce. After several washes with Tris-buffered saline containing Tween-20, the membrane was probed with horseradish-peroxidase-conjugated secondary antibody (GE Healthcare, 1:5000 dilution), washed repeatedly, and bound antibodies were detected using ECL-plus (GE Healthcare) and visualized using LAS3000 (Fujifilm, Tokyo, Japan). After antigen detection, the antibody on the membrane was stripped using WB stripping solution (Nacalai Tesque) and the membrane was re-probed with another antibody.

IgG Purification

Mouse IgG was purified using the KAPTIV-GY resin (Technogen). Briefly, approximately 0.5 mL of the KAPTIV-GY resin was loaded onto an empty column (Bio-Rad) and was washed with loading buffer (50 mmol/L bis-Tris, pH 6.8). Sera from mice at 7 weeks after immunization were applied onto the column and the column was washed with loading buffer. IgG then was eluted with glycine-HCl (pH 3.0) and the eluates were neutralized with Tris-HCl (pH 9.0). The eluted IgG (iHCV-IgG) was concentrated using an Amicon Ultra-15 (molecular weight cut-off 30,000) and IgG purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequent Coomassie brilliant blue staining. Control mouse IgG was prepared similarly from saline-injected mice (Cont-IgG).

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Supplementary Table 1. Composition of Purified HCV Particles After Ultracentrifugation

	Volume, mL	Core, pmol	RNA, copies	Protein, mg	Infectivity, FFU/mL
Culture supernatant	5720	164.0 (100%)	5.0×10^{11} (100%)	4.2×10^{3} (100%)	3.0×10^{4} (100%)
Sucrose cushion purification	1.00	106.5 (65.0%)	2.5×10^{11} (49.5%)	1.4 (0.03%)	7.2×10^{7} (41.2%)
Culture supernatant	10,500	301.7 (100%)	9.1×10^{11} (100%)	7.8×10^{3} (100%)	3.0×10^{4} (100%)
Sucrose gradient purification (Frac 6)	1.05	103.0 (34.1%)	1.8×10^{11} (19.8%)	0.7 (0.01%)	3.6×10^{7} (11.9%)
Sucrose gradient purification (Frac 8)	1.05	56.6 (18.8%)	9.0×10^{10} (9.9%)	4.3 (0.06%)	5.7×10^{7} (18.8%)

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Valine, the branched-chain amino acid, suppresses hepatitis C virus RNA replication but promotes infectious particle formation

Hisashi Ishida [a], Takanobu Kato [b], Kenji Takehana [c], Tomohide Tatsumi [a], Atsushi Hosui [a], Takatoshi Nawa [a], Takahiro Kodama [a], Satoshi Shimizu [a], Hayato Hikita [a], Naoki Hiramatsu [a], Tatsuya Kanto [a], Norio Hayashi [d], Tetsuo Takehara [a,*]

[a] Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita, Osaka, Japan
[b] Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan
[c] Exploratory Research Laboratories, Research Center, Ajinomoto Pharmaceuticals, Co, Ltd., Kanagawa, Japan
[d] Kansai Rosai Hospital, Amagasaki, Hyogo, Japan

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ABSTRACT

Background & aims: Concentrations of the branched-chain amino acid (BCAA) in the serum of patients with liver cirrhosis correlate with their liver function. Oral administration of BCAA can ameliorate hypoalbuminemia and hepatic encephalopathy. In this study, we aim to clarify the role of BCAA in regulating the replication of the hepatitis C virus (HCV).
Methods: HCV sub-genomic replicon cells, genome-length replicon cells, and cells infected with cell culture-infectious HCV (HCVcc) were cultured in media supplemented with various concentrations of BCAA, followed by evaluation of the replicon or HCV abundance.
Results: BCAA was capable of suppressing the HCV replicon in a dose-dependent manner and the effect was independent of the mTOR pathway. Of the three BCAAs, valine was identified as being responsible for suppressing the HCV replicon. Surprisingly, an abundance of HJ3-5(YH/QL), an HCVcc, in Huh7 cells was augmented by BCAA supplementation. In contrast, BCAA suppressed an abundance of HJ3-5(wild), an HCVcc that cannot assemble virus particle in Huh7 cells. Internal ribosome entry site of HCV was shown to be a target of BCAA. Single-cycle virus production assays using Huh7-25 cells, which lacked CD81 expression, revealed that BCAA, especially valine, promoted infectious virus particle formation with minimal effect on virus secretion. Thus, BCAA was found to have two opposing effects on HCV production: suppression of the HCV genome RNA replication and promotion of infectious virus formation.
Conclusions: BCAA accelerates HCV production through promotion of infectious virus formation in infected cells despite its suppressive effect on HCV genome replication.

1. Introduction

Persistent infection of hepatitis C virus (HCV) causes progressive liver disease in humans. Chronic inflammation in the liver leads to the accumulation of fibrosis and an eventual progression to liver cirrhosis. In patients with decompensated liver cirrhosis, a change in plasma amino acid composition is frequently observed. In particular, the ratio of branched-chain amino acid (BCAA) to aromatic amino acid (AAA), known as Fischer's ratio, decreases as the liver function deteriorates [1]. In such cirrhotic patients, hypoalbuminemia occurs, and it has been shown that oral administration of BCAA can ameliorate hypoalbuminemia and hepatic encephalopathy.

Three amino acids valine, leucine, and isoleucine are BCAAs, which are considered to be essential for protein anabolism. In addition to the role of acting as nutrient substrates, recent studies have demonstrated that BCAA also serve as physiologically active substances. BCAA have been shown to have pharmacological effects, such as induction of protein synthesis [2] and glucose metabolism [3]. In rat primary hepatocytes, albumin synthesis is significantly increased by BCAA administration, which is dependent on activation of the mammalian target of rapamycin (mTOR), mainly induced by leucine [4].

HCV replication is controlled by intracellular signaling pathways. In addition to the interferon (IFN)-induced JAK/STAT pathway, which activates interferon-stimulated genes, leading to strong anti-viral activity, activation of ERK [5], PI3 kinase/Akt [6,7], smad [8], PKC [9], and p38 [10], have been shown to be capable of regulating HCV replication. mTOR, one of the downstream molecules of Akt, phosphorylates the two substrates p70 S6 kinase and eukaryotic translation initiation factor 4E binding protein 1

* Corresponding author. Address: Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan. Fax: +81 6 6879 3629.
E-mail address: takehara@gh.med.osaka-u.ac.jp (T. Takehara).

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(4EBP1). p70 S6 kinase phosphorylates ribosomal S6 protein, resulting in an increase of the protein synthesis complex. Phosphorylated 4EBP1 results in its dissociation from the eukaryotic translation initiation factor 4E (eIF4E), which consequently enables eIF4E to regulate the translation initiation. Thus, together, p70 S6 kinase and 4EBP1 are responsible for the mTOR-dependent regulation of cellular translation. Moreover, both have been demonstrated to be involved in the regulation of HCV replication [6].

The finding that BCAA, per se, can activate signaling pathways suggests that they may affect HCV replication, presumably via the activation of the mTOR pathway. However, to date, no detailed investigation has been reported. Therefore, we attempt to clarify whether BCAA have a role in regulating HCV replication by using the HCV replicon system and cell culture of infectious-HCV (HCVcc). The present study reveals that although BCAA, especially valine, suppresses HCV genome replication, they eventually promote total HCV production by accelerating viral formation.

2. Methods

2.1. Cells

The hepatoma-derived cell line Huh7 and its derivatives, Huh7.5 and Huh7-25 [11], were maintained in DMEM supplemented with 10% FCS. The HCV subgenomic replicon cell line Huh-RepSI [10], and the HCV genome-length replicon cell line 2–3 [12], both harboring the HCV-N strain (genotype 1b), were previously described. The molar ratio of the BCAA mixture was adjusted to Leu:Ile:Val = 2.0:1.0:1.2 according to data from previous studies [13]. For assays to examine the role of BCAA, cells were cultured in BCAA-deficient DMEM with 10% FCS supplemented with BCAA mixtures of various concentrations (0–2 mM).

2.2. Cell culture-infectious HCV

JFH-1 is a cell culture-infectious virus of genotype 2a as previously described [14]. HJ3-5(YH/QL) is a chimeric cell culture-infectious virus with a genome consisting of the core to NS2 sequence of genotype 1a (H77) virus placed within the background of the genotype 2a JFH-1 virus. This virus contained compensatory mutations in E1 (Y361H) and NS3 (Q1251L) [15]. These two mutations rendered the chimeric RNA highly infectious.

2.3. In Vitro transcription and transfection of synthetic RNA

Plasmid DNAs encoding HJ3-5(wild) and HJ3-5(YH/QL), a wild-type chimeric virus and a chimeric virus carrying two mutations, respectively, were linearized by *Xba*I prior to transcription. RNA was synthesized with the T7 RiboMAX Express Large Scale RNA Production System (Promega, Madison, WI, USA) following the

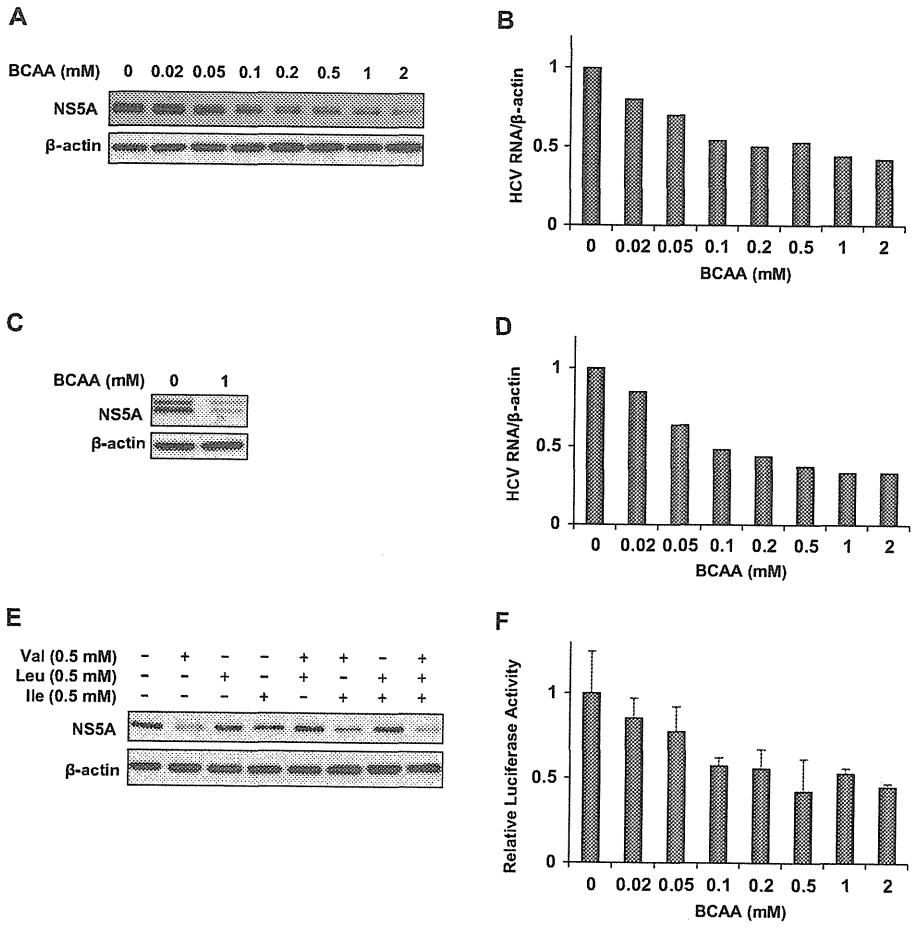

Fig. 1. BCAA limits the abundance of HCV replicon in HCV replicon cells. (A–D). Huh-RepSI (A and B) and 2–3 (C and D) cells were cultured in media for 2 days, with BCAA supplemented at concentrations of 0–2 mM. Total protein or total RNA was recovered and assayed for immunoblot (A and C) or real-time RT-PCR (B and D), respectively. (E) Three BCAAs (0.5 mM each) were added to BCAA-free culture medium of Huh-RepSI. After incubation for 2 days, immunoblot analysis of NS5A and beta-actin were performed. (F) Huh-RepSI cells were transfected with pRLHL, cultured in media with various BCAA concentrations between 0 and 2 mM. After incubation for 2 days, a dual luciferase assay was performed. The ratio of firefly luciferase activity to renilla luciferase activity was then calculated.

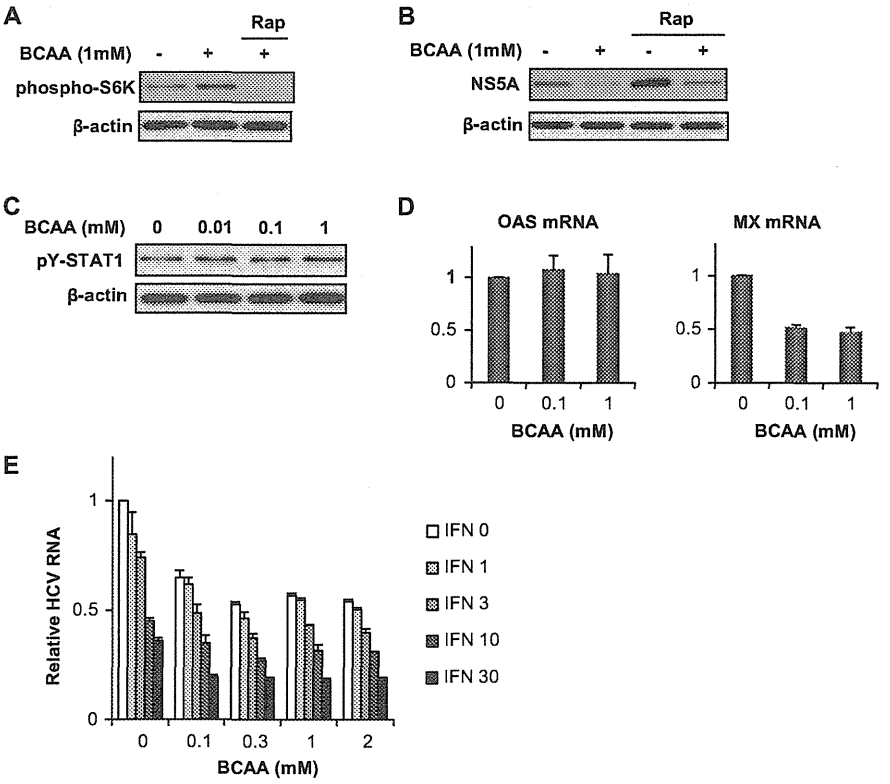

Fig. 2. BCAA-induced suppression of HCV replicon is independent of mTOR or JAK/STAT signaling. (A) Immunoblot of phosphorylated p70 S6 kinase and beta-actin in Huh-RepSI cells cultured in a medium with or without BCAA (1 mM). Rapamycin was added at 100 nM to the BCAA-containing medium. (B) Immunoblot analysis of NS5A and beta-actin in Huh-RepSI cells cultured in a medium with 1 mM BCAA or rapamycin (100 nM). (C) Huh-RepSI cells were incubated in media with various BCAA concentrations (0, 0.01, 0.1, 1 mM), and then, immunoblot analyses of phosphorylated STAT1 (Tyr701) and beta-actin were performed. (D) Huh-RepSI cells were incubated in media with various BCAA concentrations (0, 0.1, 1 mM), and then, a real-time RT-PCR analysis, for expression of OAS and MX, was performed. (E) Huh-RepSI cells were incubated in culture media with various BCAA concentrations (0–2 mM) and IFN-α (0–30 U/ml). HCV RNA abundance was normalized with beta-actin allowing the relative HCV RNA levels to be calculated, setting the HCV RNA level of 0 U/ml IFN-α and 0 mM BCAA as 1. Rap: rapamycin.

manufacturer's suggested protocol. For electroporation, Huh7 cells were washed twice with ice cold phosphate-buffered saline (PBS) and resuspended at a concentration of 10^7 cells/ml in PBS. Subsequently, 10 μg of RNA was mixed with 500 μl of the cell suspension in a cuvette, with a gap width of 0.2 cm (GenePulser II System; Bio-Rad, Hercules, CA, USA). The mixture was immediately subjected to two pulses of current with the intensities of 1.2 kV, 25 μF, and maximum resistance. Following a 10-min incubation at room temperature, the cells were transferred into growth medium.

2.4. Titration of HCV infectivity

Huh-7.5.1 cells were seeded in 96-well plates at a density of 1×10^4 cells per well 24 h prior to culture media inoculation of the HCV infected cells. Three days after infection, HCV-positive cells were detected with mouse monoclonal antibody that recognized core proteins stained with an Alexa Fluor 488 anti-mouse secondary antibody (Invitrogen, Carlsbad, CA, USA). The infectivity titer was expressed as focus-forming units per mL of supernatant (ffu/mL), expressing the mean number of HCV core-positive foci. The intracellular infectivity and specific intracellular infectivity titer were determined as described previously [16].

3. Results

3.1. BCAA suppresses the amount of HCV replicon

To investigate the role of BCAA in HCV replication, we first examined the effect of BCAA on the HCV replicon. An HCV subgenomic replicon cell line, Huh-RepSI, was incubated in culture medium that contained various concentrations of BCAA (0–2 mM) for 2 days. HCV replicon RNA, as well as the amount of protein, was suppressed by adding BCAA in a dose-dependent manner (Fig. 1A and B). To confirm the effect of BCAA, another replicon cell line, 2–3, carrying a genome-length HCV replicon, was used. In this experiment, suppression of the replicon by BCAA was observed, which is in agreement with the Huh-RepSI assay (Fig. 1C and D). This activity suggested that BCAA possessed the ability to suppress HCV replication.

Three BCAAs exist: valine, leucine, and isoleucine. As previously demonstrated, leucine contains the biological activity to activate mTOR. In addition, we showed that mTOR, which is activated by PI3 kinase/Akt, was able to suppress HCV replication [6]. Therefore, it is possible that the BCAA-mediated suppression of HCV replication was due to leucine. To test this hypothesis, the three amino acids were added independently to BCAA-deficient medium while monitoring the HCV replication level. Unexpectedly, the result refuted the hypothesis (Fig. 1E). Compared to the cells cultured in BCAA-deficient medium, supplementation with only valine efficiently suppressed the HCV replicon, whereas leucine did not; instead, it caused a slight increase. This result showed that BCAA, especially valine, but not leucine, have a suppressive effect on HCV replication.

3.2. BCAA suppresses HCV IRES activity

HCV replication can be controlled by HCV specific translation regulated by IRES, the 5′ UTR region of HCV. Therefore, we next

investigated the effect of BCAA on HCV IRES activity. To do this, we utilized a dicistronic vector, pRLHL, which consists of firefly luciferase driven by HCV IRES and renilla luciferase, translated in a cap-dependent manner (Sup. Fig. 1). Relative HCV IRES activity was evaluated using the ratio of IRES-specific luciferase activity to the cap-dependent luciferase activity. As shown in Fig. 1F, HCV IRES activity was suppressed by BCAA in a dose-dependent manner, which is similar to the result of the replicon abundance (Fig. 1A and B). Thus, the BCAA-mediated suppression of HCV replication is likely due to the inhibition of HCV IRES activity.

3.3. BCAA-mediated suppression of HCV replicon is independent of the mTOR and JAK/STAT pathways

Previous reports have demonstrated that BCAA is capable of activating mTOR [4], and we have reported that mTOR suppresses HCV replication [6]. Therefore, we examined the contribution of mTOR activation on BCAA-mediated suppression of the HCV replicon. Administration of BCAA efficiently phosphorylated p70 S6 kinase, whereas rapamycin completely inhibited its phosphorylation (Fig. 2A). Despite rapamycin enhancing the amount of HCV replicon, BCAA could efficiently suppress it, even in rapamycin-containing medium (Fig. 2B), suggesting that the suppression of the HCV replicon by BCAA is independent of mTOR activation.

The IFN-JAK/STAT signal is known to be an anti-virus pathway, induced under the condition of virus infection. HCV replication is efficiently inhibited by interferon. Therefore, we examined whether BCAA could modify the IFN signal. First, we performed an immunoblot analysis and evaluated the status of STAT1 activation, in the presence or absence of BCAA. However, the phosphorylated STAT1 level was not altered by BCAA in Huh-RepSI cells, and ISG induction was not observed; instead, the expression level of Mx was suppressed by BCAA (Fig. 2C and D). A previous study showed that rapamycin diminished the suppressive effect of IFN-α toward HCV replication via the suppression of ISG induction [17]. Subsequently, we examined the HCV replicon abundance in cells that were cultured in media with various concentrations of BCAA and IFN-α stimuli. Even with the depletion of BCAA, IFN-α efficiently and dose-dependently suppressed HCV replicon abundance. However, IFN-α-induced anti-HCV activity was not augmented by BCAA supplementation (for example, the replicon RNA level decreased to approximately 30% in both BCAA-depleted medium and 2 mM BCAA-supplemented medium) (Fig. 2E). Consequently, BCAA did not influence JAK/STAT activation, and therefore, the suppression of HCV replicon by BCAA may have been independent of the IFN-α-induced signaling pathway.

3.4. BCAA enhances HCVcc production

Next, we examined the impact of BCAA on HCVcc, a system retaining the entire HCV life cycle in a cultured cell. Here, we used HJ3-5(YH/QL), a chimeric HCV of genotype 1a (H77) and 2a (JFH-1). Surprisingly, the results of HJ3-5(YH/QL) were opposite to that of the HCV replicon: HCV abundance was upregulated in a BCAA dose-dependent manner (Fig. 3A). The HCV replicon contains NS3 to NS5B proteins, which are required for HCV RNA genome replication, but not core, E1 and E2 proteins, which are structural proteins required for viral particle formation. The discrepancy in the results between HCV replicon cells and HCVcc-infected cells might be due to differences in virus particle production.

To investigate this discrepancy, we used the wild-type HJ3-5, designated as HJ3-5(wild). As described in the Methods section, HJ3-5(YH/QL) or the HCVcc used in this study, carries two amino acid substitutions at amino acid 361 and amino acid 1251, within E1 and NS3, respectively. These two mutations render the chimeric RNA highly infectious [15]. However, without these mutations, virus particle assembly and consequent virion release from the cells to the medium would not occur. This process is thought to be due to impaired association of the HCV proteins originating from different genotypes, whereas there is no apparent change in the HCV RNA replication level in the cells [15].

We introduced the *in vitro* transcribed genome RNA of HJ3-5(wild) or HJ3-5(YH/QL) into Huh7 cells with electroporation, and then, we examined the effect of BCAA on the cell line. Normally, synthesized HCV RNA introduced into cells executes replication by utilizing HCV proteins encoded in the genome and host factors, resulting in a robust increase that is detectable after 2–3 days. BCAA decreased the abundance of HJ3-5(wild), which was similar to their effect on the HCV replicon (Fig. 3B and C). Thus, HJ3-5(wild), a virus that is defective in virus particle formation, revealed the opposite reaction to BCAA compared to the virus HJ3-5(YH/QL), a virus that is competent in virus particle formation. Together, these findings revealed that although BCAA had the ability to suppress the HCV genome replication, they promoted viral production by enhancing other steps, which included virus assembly, virus particle release and cell re-infection.

3.5. BCAA promotes infectious HCV particle formation, not virus secretion

To further assess the BCAA intracellular mechanisms that influence the HCV life cycle, we adopted a single-cycle virus production assay [18]. We used Huh7-25 cells due to the lack of surface expression of one of the cellular HCV receptors, CD81, thus being non-permissive to HCV infection. Because HCV genome replication or virus production is intact in Huh7-25, we can evaluate viral replication and secretion without the influence of re-infection.

First, we studied the replication levels of the infectious virus, JFH-1, in Huh7-25 cells. The full length of the JFH-1 genome RNA

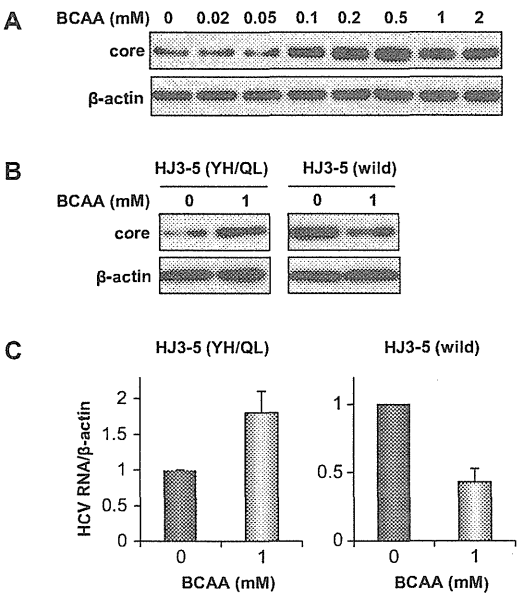

Fig. 3. HCVcc abundance was increased by BCAA. (A) HCVcc-infected Huh7 cells were cultured in media with various BCAA concentrations (0–2 mM). After incubation for 2 days, and an immunoblot analysis of core and beta-actin was performed. (B and C) Synthesized HCV genome RNA of HJ3-5 (YH/QL) or HJ3-5 (wild) was transfected into Huh7 cells via electroporation. After incubation for 24 h, cells were split into 6-well plates and incubated for 2 days in a culture medium with or without 1 mM BCAA. After the cells were harvested, immunoblot analysis of core and beta-actin (B) and real-time RT-PCR analysis (C) were performed.

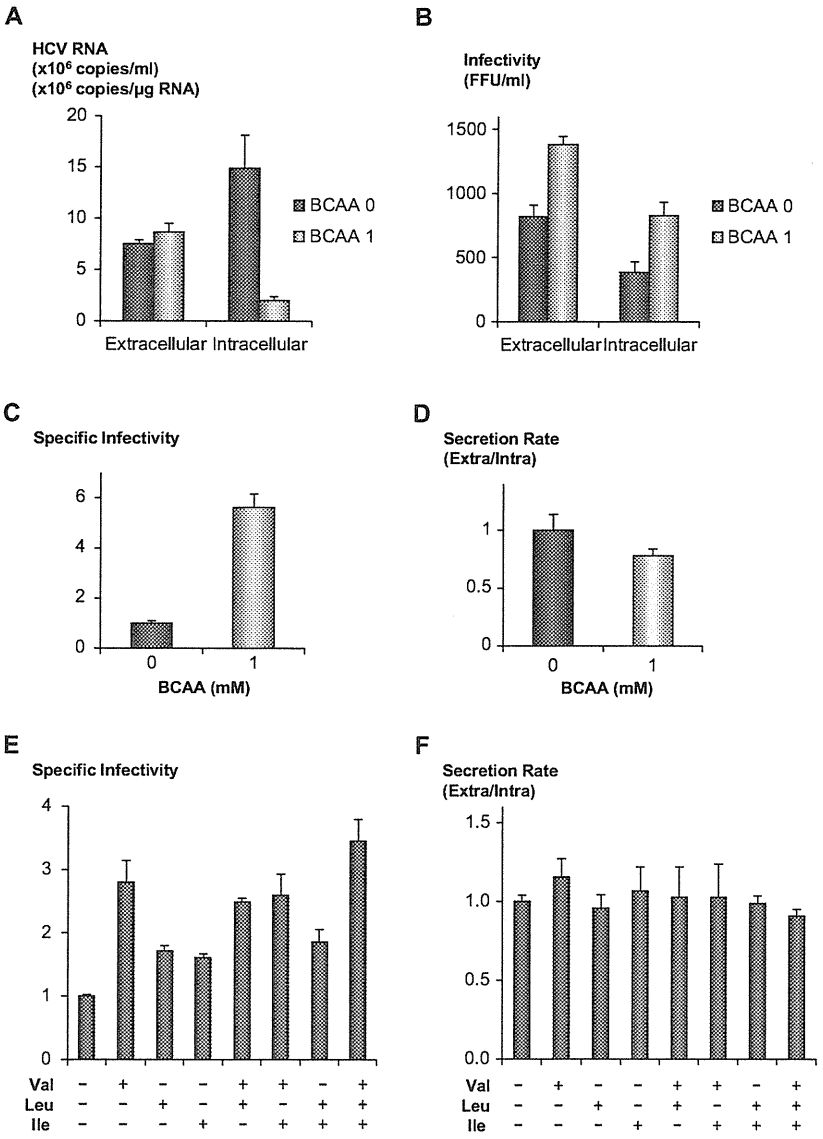

Fig. 4. Single-cycle virus production assay indicates a promoting effect of BCAA on virus formation. (A) Huh7-25 cells were transfected with *in vitro*-transcribed RNA of JFH-1, incubated in media with or without BCAA, followed by the RNA levels in the media or in the cells being calculated using the real-time quantitative RT-PCR method. (B) Infectivities in the media or in the cell lysates were measured. (C) Specific infectivities were calculated by dividing the infectivities by the HCV RNA amounts. (D) Secretion rates were calculated by dividing extracellular infectivities by intracellular infectivities. The data were presented as ratios defining the value of BCAA at 0 mM as 1. (E and F) Specific infectivities and secretion rates in the presence of valine (0.5 mM), leucine (0.5 mM), or isoleucine (0.5 mM). The data were presented as ratios defining the value with no BCAA as 1.

was translated *in vitro* and transfected into the Huh7-25 cells. The cells were cultured in media, with or without 1 mM of BCAA, with the RNA levels being monitored using quantitative RT-PCR. As observed in the experiment of replicon cells or virus particle formation-deficient viruses, the intracellular RNA level of HCV was suppressed by the presence of BCAA (Fig. 4A). However, the levels of extracellular HCV RNA were similar. Despite the suppression of intracellular HCV RNA levels by BCAA-containing medium, the infectivity titer of the intracellular virus in the cells treated with 1 mM BCAA was significantly higher than that of the cells with 0 mM BCAA (Fig. 4B). Extracellular infectivity titers were similar to those of intracellular infectivity. The specific infectivity of intracellular virus was calculated by dividing the infectivity titer by the HCV RNA level and this revealed that cultivation of the cells in a medium of 1 mM BCAA resulted in a 5.6-fold higher specific virus infectivity than that of 0 mM BCAA (Fig. 4C). Next, we measured virus secretion rates by dividing extracellular infectivity titers by intracellular infectivity titers. There was a minimal difference between infectious virus particle secretions (Fig. 4D). Thus, these results indicated that the infectious virion production was promoted in the BCAA-supplemented medium, although the virus RNA replication was suppressed.

In the study using replicon cells, valine was shown amino acid responsible for regulating HCV RNA replication (Fig. 1E). Finally, we assessed the effect of individual BCAA on virus production. HCV infected cells were cultured in media containing each amino acid at 0.5 mM or a combination of them and subsequently specific infectivity and secretion rate were examined (Fig. 4E and F). Among the three BCAAs, valine promoted infectious virus production most effectively, while leucine and isoleucine promoted infectious virus production modestly. Secretion rates did not show a significant difference.

4. Discussion

In the present study, we investigated the role of BCAA in the HCV life cycle and discovered that these amino acids suppress HCV genome replication but promote viral particle formation. Thus far, many reports have indicated that various cellular factors are involved in the regulation of HCV. In particular, intracellular signaling pathways are important modulators for HCV genome replication [5–10]. BCAA, specifically leucine, were demonstrated to have a role in activating the mTOR pathway, leading to protein synthesis such as upregulation of albumin [4] and HGF production [19]. Recently, mTOR was reported to be involved in IFN-α signaling [17]. IFN-α induced phosphorylation of STAT1 was diminished by rapamycin (but not by LY294002, a PI3 kinase inhibitor). Consequently, rapamycin inhibited the IFN-stimulated regulatory element. Although we demonstrated that BCAA can activate mTOR (Fig. 2A), the inhibition of mTOR revealed that it was not the main pathway for the BCAA suppression of HCV replication. BCAA supplementation did not change the STAT1 phosphorylation status, nor did it induce ISG expression, indicating that the JAK/STAT pathway was not relevant for the suppression of HCV replication. Considering that leucine, the factor required for mTOR activation, did not actually take part in regulation of the HCV replicon (Fig. 1E), it was not surprising that mTOR was shown to not be the responsible molecule.

Very recently, Honda et al. demonstrated that STAT1 phosphorylation was increased by BCAA in a dose-dependent manner [20]. They showed that BCAA increased the phosphorylation levels of STAT1, Foxo3a and p70 S6 kinase leading to downregulation of Socs3 expression and HCV replication. The range of BCAA concentration examined in the present study was between 0 and 2 mM. We ranged the concentration of BCAA between 0 and 2 mM because its concentration in blood is approximately 1.6 mM after oral administration of 5 g of BCAA. However, in the Honda et al. study, the BCAA concentration at which STAT1 was efficiently phosphorylated was more than 4 mM, whereas at 2 mM or less, no obvious increase in phospho-STAT1 was observed. Therefore, we may have detected no change in phospho-STAT1 due to BCAA levels used in this study. Thus, BCAA may be capable of suppressing HCV genome replication at a low concentration by inhibiting HCV IRES activity while decreasing virus replication by augmentation of IFN signaling at high concentrations.

Although BCAA suppressed replication of HCV replicon, they increased HCVcc production in infected cells. The life cycle of HCV has many steps that are required to achieve infection, such as attachment to the cell surface, endocytosis of the virus, uncoating and releasing genome RNA, RNA replication, polyprotein synthesis and processing, viral assembly, and release of progeny virus. Among these, the HCV replicon system only represents the steps of genome RNA replication and non-structural protein synthesis in the cells, and BCAA affects these by impairing protein synthesis via suppression of HCV IRES activity. However, HCVcc replication requires all of these steps. We assumed that the increase of HCVcc due to BCAA indicated that some step(s) must be upregulated by BCAA to the extent of overcoming the decreased genome replication. The study of particle formation-deficient viruses suggested that virus assembly or some downstream step in the virus life cycle was critical for the augmentation of HCVcc by BCAA. A single-cycle virus production assay indicated that the production of an infectious virus was prominent in the presence of BCAA, while virus secretion was not strongly affected. Although HCV genome replication was suppressed by BCAA, more infectious virus particles were secreted into the media, and they could have re-infected the Huh7 cells. We suggest that the abundant infectious HCV in BCAA-supplemented medium causes amplification of the virus during repetition of such re-infection, which leads to an accumulation of HCV in the cells, and thus, the abundance of HCV RNA in the cells with BCAA medium overcomes that without BCAA. Further investigation is needed on the detailed mechanisms defining how BCAA regulates HCV particle formation. Clarification of this process could contribute to new insights into HCV replication and could also suggest a basis for treatment of HCV patients.

Acknowledgments and disclosures

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.06.051.

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1(OH) Vitamin D3 Supplementation Improves the Sensitivity of the Immune-Response during Peg-IFN/RBV Therapy in Chronic Hepatitis C Patients-Case Controlled Trial

Yasuteru Kondo[1]*, Takanobu Kato[2], Osamu Kimura[1], Tomoaki Iwata[1], Masashi Ninomiya[1], Eiji Kakazu[1], Masahito Miura[3], Takehiro Akahane[4], Yutaka Miyazaki[5], Tomoo Kobayashi[6], Motoyasu Ishii[7], Norihiro Kisara[7], Kumiko Sasaki[7], Haruo Nakayama[8], Takehiko Igarashi[9], Noriyuki Obara[10], Yoshiyuki Ueno[11], Tatsuki Morosawa[1], Tooru Shimosegawa[1]

1 Division of Gastroenterology, Tohoku University Hospital, Sendai City, Japan, **2** Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan, **3** Department of Gastroenterology, South Miyagi Medical Center, Oogawara Town, Japan, **4** Department of Gastroenterology, Ishinomiki Red Cross Hospital, Ishinomaki City, Japan, **5** Department of Gastroenterology, Kosai Hospital, Sendai City, Japan, **6** Department of Gastroenterology, Rosai Hospital, Sendai City, Japan, **7** Department of Gastroenterology, Miyagi Shakai Hoken Hopital, Sendai City, Japan, **8** Department of Gastroenterology, Iwaki Kyoritsu Hosptal, Iwaki City, Japan, **9** Department of Gastroenterology, Oosaki Citizen Hoptial, Oosaki City, Japan, **10** Department of Gastroenterology, Iwate Central Hospital, Morioka City, Japan, **11** Department of Gastroenterology, Yamagata University School of Medicine, Yamagata City, Japan

Abstract

Objective: 1,25(OH)$_2$ vitamin D3 can affect immune cells. However, the mechanism responsible for the favorable effects of 1(OH) vitamin D3, which becomes 1,25(OH)$_2$ vitamin D3 in the liver, is not clear. The aim of this study is to analyze the immunological response of 1(OH) vitamin D3 supplementation in CH-C patients.

Design: Forty-two CH-C patients were treated with 1(OH) vitamin D3/Peg-IFNα/RBV. Forty-two case-matched controls were treated with Peg-IFNα/RBV. The expression of Interferon-stimulated genes (ISGs)-mRNA in the liver biopsy samples and JFH-1 replicating Huh-7 cells were quantified by real-time PCR. Ten kinds of cytokines in the plasma were quantified during treatment by using a suspension beads array. A trans-well co-culture system with peripheral blood mononuclear cells (PBMCs) and Huh-7 cells was used to analyze the effect of 1(OH) vitamin D3. The activities of the Th1 response were compared between subjects treated with 1(OH) vitamin D3/Peg-IFN/RBV and those treated with Peg-IFN/RBV therapy alone.

Results: 1(OH) vitamin D3/Peg-IFN/RBV treatment could induce rapid viral reduction, especially in *IL28B T/T* polymorphism. Several kinds of cytokines including IP-10 were significantly decreased after 4 weeks of 1(OH) vitamin D3 treatment ($p<0.05$). Th1 responses in the subjects treated with 1(OH) vitamin D3/Peg-IFN/RBV were significantly higher than those treated with Peg-IFN/RBV at 12 weeks after Peg-IFN/RBV therapy ($p<0.05$). The expression of ISGs in the patient's liver biopsy samples was significantly lower than in those treated without 1(OH) vitamin D3 ($p<0.05$).

Conclusion: 1(OH) vitamin D3 could improve the sensitivity of Peg-IFN/RBV therapy on HCV-infected hepatocytes by reducing the IP-10 production from PBMCs and ISGs expression in the liver.

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* E-mail: yasuteru@ebony.plala.or.jp

Introduction

Hepatitis C Virus (HCV) is a non-cytopathic virus that causes chronic inflammation, fibrosis and hepatocellular carcinoma (HCC) [1]. Recently, it has been reported that vitamin D3 supplementation could improve the SVR in chronic hepatitis C (CH-C) patients [2,3]. Moreover, the amount of 25-hydoxyvitamin D3 (25(OH) vitamin D3) in the serum could affect the treatment response to pegylated interferon α (Peg-IFN-α)/ribavirin (RBV) therapy and is complementary to interleukin 28B (*IL-28B*) rs1297860 C/T polymorphism in enhancing the correct prediction of the SVR in CH-C [4]. Another group reported that, in patients with genotype 1 HCV persistent infection, the 25(OH) vitamin D serum levels and *IL28B* polymorphism were independently associated with the likelihood of achieving a rapid viral response and SVR after treatment with Peg-IFN/RBV [5].

Although several kinds of mechanisms for the favorable effects of vitamin D3 supplementation were reported, the total effect of vitamin D3 supplementation remains unclear [6,7]. One group reported that 25(OH) vitamin D3, but not vitamin D3 or 1, 25 dihydoxyvitamin D3 (1, 25$(OH)_2$ vitamin D3), appeared to inhibit the viral life cycle at the level of infectious HCV assembly [7]. Another group reported that vitamin D3 or 1,25$(OH)_2$ vitamin D3 and IFN-α could synergistically inhibit HCV production by enhancing the IFN signaling pathway [6]. However, the effect of vitamin D3 on the adaptive immune system in CH-C patients has not been reported yet.

It has been reported that vitamin D3, as synthesized in the skin by photolysis from 7-dehydrocholesterol or ingested with food, is transported in the blood to the liver where it is hydoxylated at the C-25-position [8]. Then, it is hydoxylated at the C-1α-position to form the active metabolite 1,25 $(OH)_2$ vitamin D3 in the kidney [9,10]. In this study, we selected 1(OH) vitamin D3, since the local concentration in the liver should be higher than other metabolites of vitamin D3. Moreover, 1 (OH) vitamin D3 is safe and commonly used in worldwide. 1,25 $(OH)_2$ vitamin D3 is known to regulate calcium and phosphorus metabolism in skeletal homeostasis [11]. It has been reported that 1,25$(OH)_2$ vitamin D3 plays an important role as an immune-modulator targeting various immune cells [12–15]. Various kinds of immune cells express not only vitamin D receptors (VDRs) but also vitamin D-activating enzymes, allowing local conversion of inactive vitamin D into 1,25 $(OH)_2$ vitamin D3 within the immune system [16,17]. The active metabolite1,25$(OH)_2$ vitamin D3 could enhance the anti-mycobacterial activity in monocytes by enhancing the chemotactic and phagocytic capacity of macrophages [18]. Moreover, 1,25$(OH)_2$ vitamin D3 might play an important role in the binding and capturing of antigens by dendritic cells (DCs) at the initiation of the immune response [19]. On the other hand, some groups reported that 1, 25$(OH)_2$ vitamin D3 could inhibit the differentiation and maturation of DCs [19,20]. In addition to monocyte-derived cells, $CD3^+$ T cells, $CD19^+$ B cells, natural killer cells (NK cells) could be directly and/or indirectly affected by 1, 25$(OH)_2$ vitamin D3[12,17,21–24]. It has been reported that 1, 25$(OH)_2$ vitamin D3 could contribute to the suppression of the immune response in autoimmune diseases [14,15,25]. More Recently, the expression of specific VDRs in liver cells and reduced expression of VDRs in CH-C patients have been reported [26]. In addition, an inverse relationship between the liver VDR expression and inflammation severity has been found [26]. However, the effects of 1, 25$(OH)_2$ vitamin D3 for the adaptive immune system in the condition of CH-C patients and during treatment with peg-interferon α and ribavirin (Peg-IFN/RBV) are still unclear. Therefore, it is urgent to analyze the effect of 1, 25$(OH)_2$ vitamin D3 on the adaptive immune responses that could contribute to the outcome of Peg-IFN/RBV therapy.

Materials and Methods

Study Design and Patients

Multi-centers that belong to the Tohoku-liver-study-group (TLG) were involved in this study. Dr. Abu-Mouch et al. reported that the SVR rate of Peg-IFN/RBV plus Vitamin D treatment group was 86% in the AASLD 2009 annual meeting [27]. On the

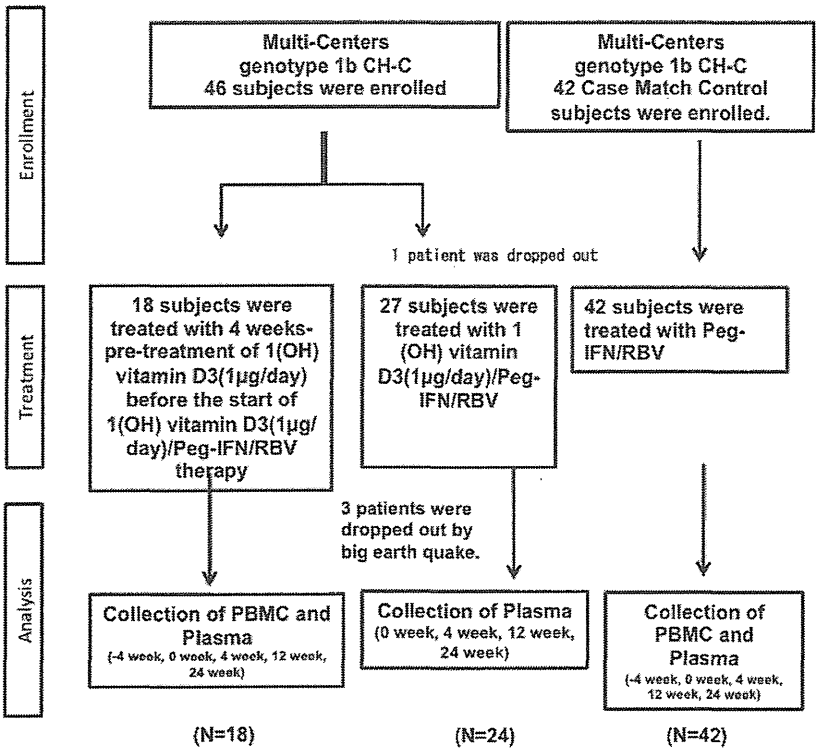

Figure 1. Enrollment of CH-C patients. 46 patients with genotype 1b and high viral loads were enrolled in this study. In total, 4 patients were dropped from this study.
doi:10.1371/journal.pone.0063672.g001

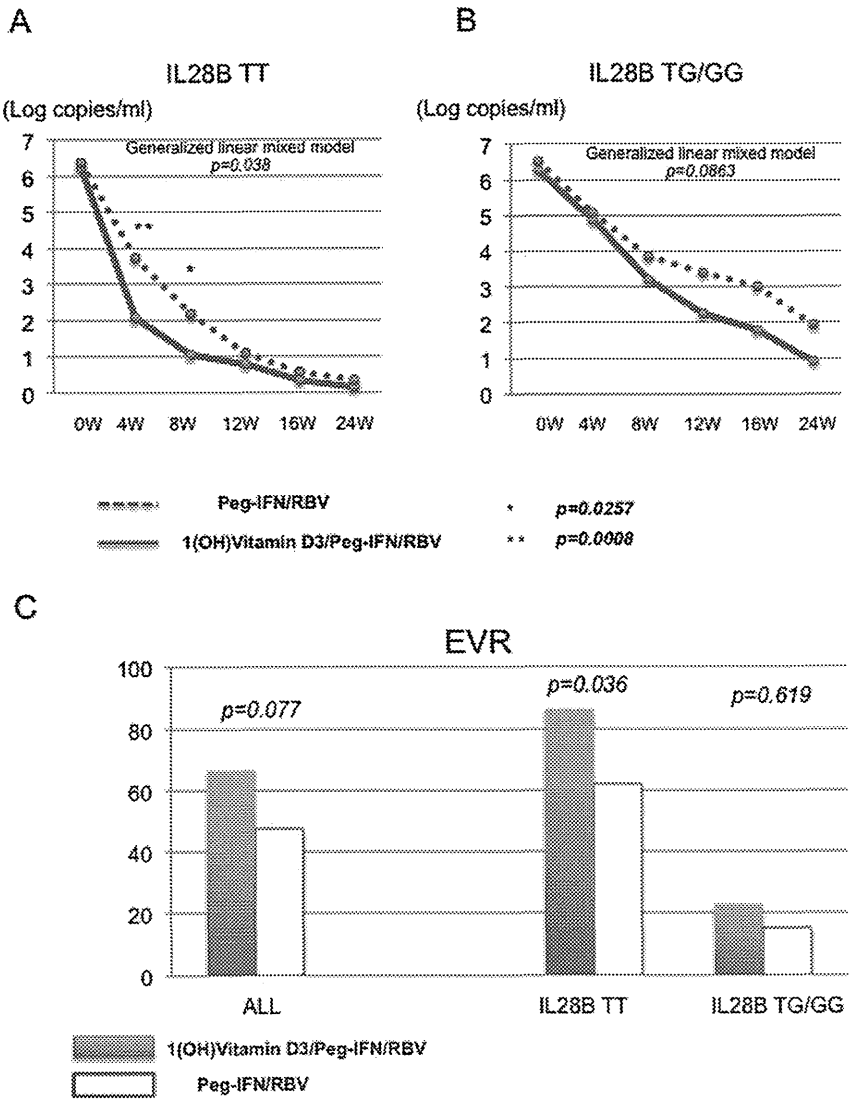

Figure 2. Comparison of viral dynamics and treatment response. Viral dynamics of subjects with IL28B T/T major homo polymorphism are shown (A). Viral dynamics of subjects with IL28B T/G or G/G minor polymorphism are shown (B). Blue lines indicate viral dynamics of subjects treated with 1(OH) Vitamin D3/Peg-IFN/RBV. Dotted lines indicate viral dynamics of subjects treated with Peg-IFN/RBV. *p<0.05 **p<0.01 The rates of early virological response in the patients treated with 1(OH) vitamin D3/Peg-IFN/RBV and Peg-IFN/RBV are shown (C).
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other hand, the SVR rate of Peg-IFN/RBV treatment group was 42%. Considering the uncertainty, we speculated that the EVR rate might be 90% of the EVR rate in the Peg-IFN/RBV plus Vitamin D treatment group because the reported EVR rate in this study was remarkably high. Based on the results of this study, we enrolled about 80 patients including control patients: there was 10% loss in the proportion of patients during the 48 weeks therapy ($\alpha = 0.05$, statistical power 90%) (EVR rate 77% vs 42%). The alfa level was two-sided. Forty-six CH-C (Genotype 1b) patients were enrolled in this study (Fig. 1). Forty-two matched historical controls treated with Peg-IFN-α/RBV therapy were analyzed. The inclusion criteria were as follows: age between 20 and 75 years, high viral load (>5.0 log copies/mL) by real time PCR analysis of HCV-RNA, absolute white blood cell count >2,000/ml, neutrophil count >1,000/ml, platelet count >90,000/ml, and hemoglobin concentration >11 g/dL in laboratory tests. The exclusion criteria were as follows: other liver diseases, including autoimmune hepatitis and alcoholic hepatitis, decompensated liver cirrhosis, liver failure, severe renal disorders, abnormal thyroid function, poorly controlled diabetes, poorly controlled hypertension, medication with immune-modulators, interstitial pneumonia and severe depression. Permission for the study was obtained from the Ethics Committee at Tohoku University Graduate School of Medicine (permission no. 2010-114) (UMIN000003694). The date of the protocol fixation was 10th June 2010. The anticipated trial start date was 11th June 2010. Patients in the 1(OH) vitamin D3/Peg-IFN/RBV group were treated from June 2010 to June 2012. Patients in the Peg-IFN/RBV group were treated from March

2009 to June 2012. Liver biopsy samples of the historical control were from previous studies (Permission no. 2009-166) (UMIN000002326), (Permission no. 2009-209), and (Permission no. 2010-404). Written informed consent of the control subjects treated with Peg-IFN/RBV treatment was obtained in the previous study and in the present study (Permission no. 2009-166) (UMIN000002326), (Permission no. 2009-209), and (Permission no. 2010-404). Written informed consent was obtained from all the participants enrolled in the 1(OH) vitamin D3/Peg-IFN/RBV treatment group. Participants were monitored for a year. At each assessment, patients were evaluated by hematological test, biochemical laboratory tests, immunological test and virological tests. Liver histology was analyzed at the start of Peg-IFN/RBV therapy using the METAVIR score.

Detection of IL-28B Polymorphism

Genomic DNA was isolated from peripheral blood mononuclear cells (PBMCs) using an automated DNA isolation kit. Then, the polymorphism of *IL28B* (rs8099917) was analyzed using real-time polymerase chain reaction (PCR) (TaqMan SNP Genotyping Assay, Applied Biosystems, CA, USA). Detection of the *IL28B* polymorphism was approved by the Ethics Committee at Tohoku University Graduated School of Medicine (permission no. 2010-323).

Isolation of Peripheral Blood Mononuclear Cells (PBMCs), $CD4^+$ Cells and Cell Culture

PBMCs were isolated from fresh heparinized blood by means of Ficoll-Hypaque density gradient centrifugation (Amersham Bioscience, Uppsala, Sweden). Primary $CD4^+$ cells were isolated using magnetic beads (Dynal). PBMCs were used to analyze the effect of the metabolite of α-calcidol(1(OH) vitamin D3) without direct cell to cell contact in an Huh-7 cells-transwell system. PBMCs and Huh-7 cells were cultured with serum-free complete medium that were previously made by our group [28]. A thousand times higher amount of 1(OH) vitamin D3 was used to analyze the effect of 1,25 $(OH)_2$ vitamin D3, which comes from the lower part of chamber, since the Huh-7 cells have several enzymes that could convert 1(OH) vitamin D3 to 1,25 $(OH)_2$ vitamin D3. The supernatant was harvested at 48 hours after the addition of 1(OH) vitamin D3 or 1,25 $(OH)_2$ vitamin D3.

Flow Cytometry Analysis

PBMCs were stained with CD3-pacific-blue, CD4-PE/Cy7, CD25-PE, CD127-APC, CD183 (CXCR3)-APC/Cy7, CD195 (CCR5)-FITC, Viaplobe and isotype control antibodies (BD pharmingen, San Jose, CA, USA) for 15 min on ice to analyze the frequency of CD3+CD4+CXCR3+CCR5+ cells (Th1) and CD3+CD4+CD25+CD127− (Tregs) by FACSCanto-II (BD). The FCS files 3.0 were analyzed by Flowjo 7.60 software.

Multiplex Beads Suspension Array

The culture supernatant of PBMCs treated with the active vitamin D3 metabolite (1,25 $(OH)_2$ vitamin D3) and the plasma obtained from CH-C patients treated with or without alfa-calcidol (1(OH) vitamin D3) were sequentially analyzed by suspension beads array (BIO-RAD Laboratories, Tokyo, Japan). Suspension beads array was performed following the manufacturer's instruction. Briefly, the supernatant was incubated with first-antibody binding magnetic beads. Then, the detection antibody and PE

Table 1. Clinical characteristics of subjects enrolled in this study.

	PEG-IFNα/RBV	PEG-IFNα/RBV+VD3	PEG-IFNα/RBV+VD3	
	(n = 42)	(n = 42)	With Pre-VD3	Without Pre-VD3
			(n = 18)	(n = 24)
Gender(M/F)	19/23	15/27	6/12	9/15
Age	58.3(35–72)	59.1(29–71)	58.6(29–71)	58.5(43–71)
Body Weight	58.4	58.1(41.2–81)	56.4(41.2–81)	59.4(43–78)
History of IFN(+/−)	13/29	13/29	7/11	6/18
IL-28B(TT/TG,GG)	29/13	29/13	10/8	19/5
Sampling Point (week)	0W	All 0W	−4W	0W
HCV-RNA	6.3(5.1–7.2)	6.3(5.2–7.4)	6.3(5.2–7.1)	6.4(5.3–7.4)
ALT	68.5 (15–234)	66.4(16–242)	47.9(22–108)	78(16–242)
AST	55.2(16–161)	58.1(21–251)	45.3(22–112)	66.1(21–251)
WBC	5045(3050–7800)	5165(2400–9300)	5055(3100–9300)	5530(2400–8130)
RBC	441.3(355–522)	441.5(375–567)	450(375–567)	446(383–515)
PLT	16.6(9.4–29.4)	16.7(9.3–27.6)	16.6(9.3–27.6)	16.7(9.3–23.9)
Nue	2845(1750–5020)	2911(1190–7160)	2792(1190–7160)	3476(1533–5070)
Hb	13.8(11.8–15.9)	13.6(12–16.3)	13.7(12–15.2)	14.1(12.6–16.3)
Serum Ca	9.3(8.5–9.8)	9.2(8.6–10.1)	9.4(8.9–10.1)	9.2(8.6–10)
Insulin	9.4(6.8–20.2)	9.5(1.6–25.5)	9(4.76–20.8)	9.6(1.6–25.5)
T-cho	170.6(118–214)	172.4(116–227)	168.2(116–226)	173.7(119–227)
TG	108.5(55.6–210)	106.4(37–427)	118.9(37–259)	103.2(51–427)

HCV-RNA(log copies/ml), ALT(U/l), AST(U/l), WBC(counts/μl), RBC(x10^3counts/μl), PLT(x10^4counts/μl), Neut(counts/μl), Hb (g/dl), Serum Ca (mg/dl), Insulin (μU/ml), T-cho (mg/dl), TG (mg/dl).

doi:10.1371/journal.pone.0063672.t001

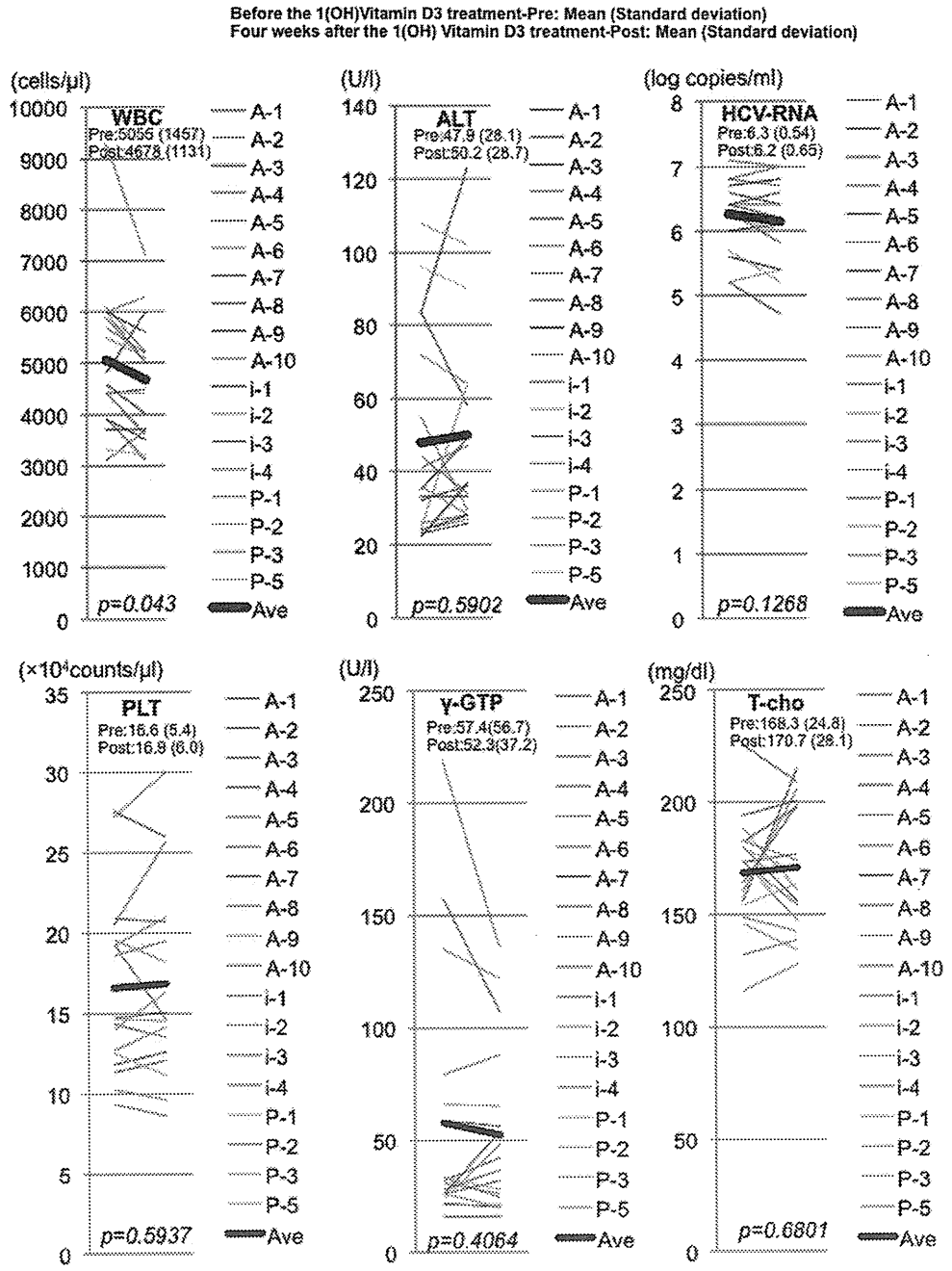

Figure 3. Comparison of hematological and biochemical analysis between before and after 4-week 1(OH) vitamin D3 treatment. Representative hematological, biochemical and virological data are shown. WBC indicates white blood cell count. ALT indicates alanine transaminase. HCV-RNA indicates titer of hepatits C virus RNA. PLT indicates platelet count. γ-GTP indicates gamma-glutamyl traspeptidase. T-cho indicates total cholesterol. The data at pre- and post-4weeks administration of 1(OH) vitamin D3 without Peg-IFN/RBV are shown. Black lines indicate the average of each analysis.

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conjugated streptavidin were reacted after the appropriate washing steps. Finally, the reaction plates were analyzed by Bio-plex 200 system.

Real-time Polymerase Chain Reaction

RNA was isolated using a Qiagen RNeasy mini kit (Valencia, CA) and the yields were determined by absorption spectroscopy using a Nano-Drop (NanoDrop Products, Wilmington, DE). After the extraction of total RNA and the reverse transcription (RT)

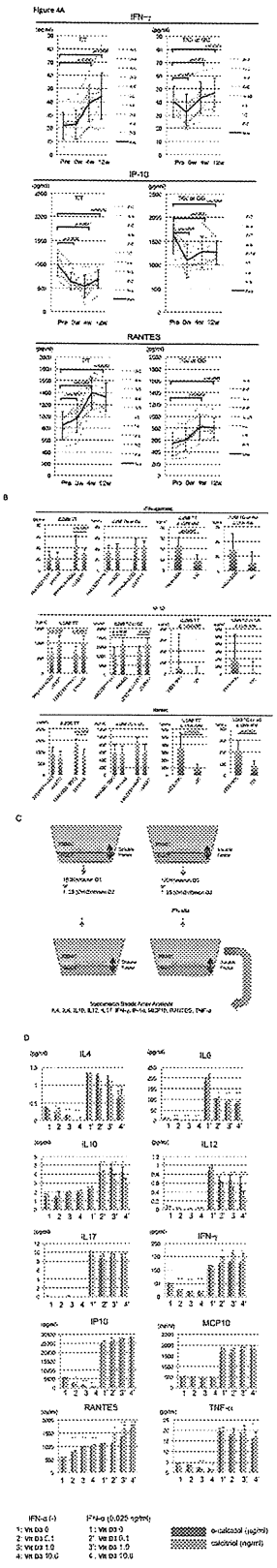

Figure 4. Cytokine profiles in the *ex vivo* and *in vitro* samples treated with vitamin D3. Sequential data of quantification of 3 cytokines (IFN-γ, IP-10 and RANTES) during 1(OH) vitamin D3 pre-treatment (pre vs 0w), 1(OH) vitamin D3/Peg-IFN/RBV therapy are shown (A). Dotted lines indicate the data of each subject. Black lines indicate the averaged data. Error bars indicate standard deviation. The data from IL28B (T/T) subjects or IL28B (T/G or G/G) subjects are shown in the independent graphs (A). Comparisons of the amounts of 3 cytokines (IFN-γ, IP-10 and RANTES) between the 1(OH) vitamin D3/PEG-IFN/RBV group (VitD3+standard of care (SOC)) and Peg-IFN/RBV group (SOC) at 0 weeks and 12 weeks after the start of Peg-IFN/RBV treatment are shown (B). Analysis of the changes in the amounts of the 3 cytokines (IFNγ, IP-10 and RANTES) during 12 weeks treatment of Peg-IFN/RBV is shown. Schema of *in vitro*-analysis of co-culture is shown (B). alfa-calcidol: 1(OH)vitamin D3 and calcitriol: 1,25(OH)vitamin D3 were used to analyze the cytokine production *in vitro*. Black bars indicate the data from samples treated with alfa-calcidol. Gray bars indicate the data from samples treated with calcitriol. *$p<0.05$.
doi:10.1371/journal.pone.0063672.g004

procedure, real-time polymerase chain reaction (PCR) using a TaqMan Chemistry System was carried out. The ready-made set of primers and probe for the amplification of IFN-γ, T-bet, Mx1 (ID Hs00895608), IFI44 (ID Hs00197427), IFIT1 (ID Hs01911452) and glyceraldehyde 3-phsphate-dehydrogenase (GAPDH) were purchased from Perkin-Elmer Applied Biosystems (Carlsbad, CA, USA). The relative amount of target mRNA was obtained by using the comparative threshold (CT) cycle method.

The Quantification of ISGs mRNA in Hepatocyte Cell Culture

Huh-7 cells were treated with ethanol (control), 1(OH) vitamin D3 (1.0 μM) or 1,25(OH)2 vitamin D3 (1.0 μM) after transfection of poly IC (Sigma-Aldrich, St. Louis, MO) or in vitro transcribed JFH-1 full-length RNA. Cells were harvested 30 hour after transfection, and the expression levels of Mx, IFI44 and IFIT1 mRNA were assessed by real-time PCR using TaqMan Gene Expression Master Mix (Applied Biosystems, Carlsbad, CA) and gene-specific primer and probe sets (TaqMan Gene Expression Assay; Applied Biosystems) in accordance with the manufacturer's instructions. The expression levels of genes with or without vitamin D3 treatment were expressed by the log fold increase of untreated Huh-7 cells.

Statistical Analysis

The data in Fig. 2A and B were analyzed using a generalized linear mixed model (Treatment group of 1(OH) vitamin D3/Peg-IFN/RBV and Peg-IFN/RBV were fixed-effect. Duration of treatment was random-effect.) and Student's t test. The data in Fig. 2C were analyzed by χ^2 test. The data in Fig. 3, Fig. 4A and Fig. 5B were analyzed by paired t test. The data in Fig. 4C were analyzed by Dunnett's test. The data in Fig. 5C were analyzed by Tukey's test. The data in Fig. 4B, Fig. 5D and Fig. 6 were analyzed by Student's t test. The cut-off of acceptance of test's results was $p<0.05$ with a confidence interval of 95%. All statistical analyses were carried out using JMP Pro version 10 (SAS Institute Inc., Cary, NC, USA).

Results

Efficacy and Tolerability of 1(OH) Vitamin D3 Combined with Peg-IFN/RBV Therapy

The characteristics of 42 patients treated with 1(OH) vitamin D3 (1 μg/day)/Peg-IFN/RBV therapy are shown in Table 1. The subjects enrolled in this study were 29 to 71 years old. 13 patients