HEPATOLOGY
Official Journal of the American Association for the Study of Liver Diseases

25-Hydroxyvitamin D_3 Suppresses Hepatitis C Virus Production

Takuya Matsumura,[1,2] Takanobu Kato,[2] Nao Sugiyama,[2] Megumi Tasaka-Fujita,[2] Asako Murayama,[2] Takahiro Masaki,[2] Takaji Wakita,[2] and Michio Imawari[1]

Because the current interferon (IFN)-based treatment for hepatitis C virus (HCV) infection has a therapeutic limitation and side effects, a more efficient therapeutic strategy is desired. Recent studies show that supplementation of vitamin D significantly improves sustained viral response via IFN-based therapy. However, mechanisms and an active molecular form of vitamin D for its anti-HCV effects have not been fully clarified. To address these questions, we infected HuH-7 cells with cell culture-generated HCV in the presence or absence of vitamin D_3 or its metabolites. To our surprise, 25-hydroxyvitamin D_3 [25(OH)D_3], but not vitamin D_3 or 1,25-dihydroxyvitamin D_3, reduced the extra- and intracellular levels of HCV core antigen in a concentration-dependent manner. Single-cycle virus production assay with a CD81-negative cell line reveals that the inhibitory effect of 25(OH)D_3 is at the level of infectious virus assembly but not entry or replication. Long-term 25(OH)D_3 treatment generates a HCV mutant with acquired resistance to 25(OH)D_3, and this mutation resulting in a N1279Y substitution in the nonstructural region 3 helicase domain is responsible for the resistance. *Conclusion:* 25(OH)D_3 is a novel anti-HCV agent that targets an infectious viral particle assembly step. This finding provides insight into the improved efficacy of anti-HCV treatment via the combination of vitamin D_3 and IFN. Our results also suggest that 25(OH)D_3, not vitamin D_3, is a better therapeutic option in patients with hepatic dysfunction and reduced enzymatic activity for generation of 25(OH)D_3. (HEPATOLOGY 2012;56:1231-1239)

Hepatitis C virus (HCV) infection affects about 200 million people worldwide.[1,2] The majority of HCV-infected patients fail to clear the virus and develop chronic liver diseases, including cirrhosis and hepatocellular carcinoma. Standard treatment for chronic hepatitis C is currently based on a combination of pegylated interferon (IFN) and ribavirin.[2] However, the therapy is accompanied by substantial side effects and is only effective in about half of patients.[3,4] Thus, it is critical to provide a new therapeutic modality against chronic hepatitis C. Recently, vitamin D supplementation has been shown to improve the efficacy of combination therapy with IFN and ribavirin.[5,6] However, mechanisms of this effect have not yet been fully elucidated.

Vitamin D absorbed in the intestine from diet or synthesized in the skin is converted to 25-hydroxyvitamin D [25(OH)D] in the liver. Released 25(OH)D is bound to α-globulin and transported to proximal tubules of the kidney,[7] where 25(OH)D is hydroxylated either by 25(OH)D-1 alpha-hydroxylase to generate the active form, 1,25-dihydroxyvitamin D [1,25$(OH)_2$D], or by 25(OH)D-24-hydroxylase to form the biologically inactive form, 24,25-dihydroxyvitamin D [24,25$(OH)_2$D]. 1,25$(OH)_2$D is a key hormone for calcium and bone homeostasis, and its production is tightly regulated by plasma levels of calcium and phosphorus and parathyroid hormone, In addition, vitamin D has nonskeletal actions, and vitamin D deficiency is associated with many diseases including cancer, autoimmune disorder, cardiovascular disease, insulin resistance, and infectious diseases.[8-13] Thus it is not surprising that vitamin D status also

Abbreviations: 1,25$(OH)_2$D, 1,25-dihydroxyvitamin D; 24,25$(OH)_2$D, 24,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; Ag, antigen; DMEM, Dulbecco's modified Eagle's medium; HCV, hepatitis C virus; HCVcc, cell culture-generated HCV JFH-1 virus; HCVpp, HCV pseudoparticles; IFN, interferon; ISG, IFN-stimulated gene; JFH-1/wt, wild-type JFH-1; MLV, murine leukemia virus; NS3, nonstructural region 3; PCR, polymerase chain reaction; WST-8, water-soluble tetrazolium salt 2-(2-methoxy-4- nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt.

From the [1]Division of Gastroenterology, Department of Medicine, Showa University School of Medicine, Tokyo, Japan; and the [2]Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan.

Supported by the Japan Society for the Promotion of Science, the Ministry of Health, Labor and Welfare of Japan, the Ministry of Education, Culture, Sports, Science and Technology, and the Research on Health Sciences Focusing on Drug Innovation from the Japan Health Sciences Foundation. T. Matsumura and M. Imawari were partly supported by Chugai Pharmaceutical Co. Ltd. (Tokyo, Japan).

affects the eradication of HCV by IFN-based therapy in patients chronically infected with the virus.[5,6]

In this study, we evaluated the anti-HCV effects of vitamin D_3 and its metabolites, $25(OH)D_3$, $1,25(OH)_2D_3$, and $24,25(OH)_2D_3$. Here we show that $25(OH)D_3$, but not $1,25(OH)_2D_3$, possesses an anti-HCV effect targeting the assembly of the infectious virus. This finding suggests the novel conjunctive role of $25(OH)D_3$ in IFN-based therapy against chronic hepatitis C.

Materials and Methods

Cell Culture and Reagents. The human hepatoma cell line, HuH-7, and its derivative cell line, Huh-7.5.1, provided by Francis Chisari (Scripps Research Institute, La Jolla, CA) were maintained at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal bovine serum. The HuH-7 derivative cell line Huh7-25, which lacks CD81 expression,[14] was also used.

Vitamin D_3 and $24,25(OH)_2D_3$ were purchased from Enzo Life Sciences Inc. (Farmingdale, NY). $25(OH)D_3$ was obtained from Immundiagnostik AG (Bensheim, Germany). $1,25(OH)_2D_3$ was kindly provided by Chugai Pharmaceutical Co. Ltd. (Tokyo, Japan). Vitamin D_3 and its metabolites were dissolved in ethanol at the stock concentration of 2.0 mM, and stored at −30°C or −80°C until use. Only glass- or Teflon-made wares were used for handling ethanol solutions of vitamin D_3 and its metabolites.

Human IFN-alfa2b was obtained from MSD K.K. (Tokyo, Japan).

Cell Culture–Generated HCV. The production of cell culture–generated HCV JFH-1 virus (HCVcc) has been reported.[15] Briefly, the plasmid pJFH-1 was linearized at the 3′ end of the full-genome JFH-1 complementary DNA by XbaI digestion. Digested plasmid DNA was purified and used as a template for *in vitro* RNA synthesis with a MEGAscript T7 kit (Ambion, Austin, TX). Synthesized full-length JFH-1 RNA was electroporated into Huh-7.5.1 cells with cytomix as described. After long-term culture of transfected cells, cell culture–adapted HCVcc was harvested and stocked for further infection studies (Kato et al., unpublished data).

Quantification of HCV RNA, Core Antigen, and Cell Viability. Total RNA was extracted from 140 μL of culture medium with the QIAamp Viral RNA kit (QIAGEN, Valencia, CA) or from harvested cell pellets with the RNeasy mini kit (QIAGEN). Real-time quantitative reverse-transcription polymerase chain reaction (PCR) was performed to determine the copy number of HCV RNA as described.[16] The concentration of total RNA in the cells was determined using a Nanodrop Spectrophotometer ND-1000 (Thermo Scientific, Rockford, IL). The concentration of HCV core antigen (Ag) in filtered culture medium and cell lysates was determined using the Lumipulse Ortho HCV Ag kit (Ortho Clinical Diagnostics, Tokyo, Japan) as described.[17]

To assess cell viability, a water-soluble tetrazolium salt, 2-(2-methoxy-4- nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt (WST-8) was used as an indicator.[18] In brief, the WST-8 reagent solution (10 μL) was added to each well of a 96-well microplate containing 100 μL of cells in the culture medium. The plate was incubated for 1 hour at 37°C, and the absorbance in each well was measured at 450 nm using a microplate reader.

HCV Pseudoparticles Assay. HCV pseudoparticles (HCVpp) harboring E1 and E2 glycoproteins of various HCV clones (H77, genotype 1a; TH, genotype 1b; JFH-1, genotype 2a; and J6CF, genotype 2a) or vesicular stomatitis virus G envelope glycoprotein were produced as described.[19,20] Briefly, to generate HCVpp, the glycoprotein-expressing vector, the gag-pol expression vector encoding murine leukemia virus (MLV) retroviral cores, and the MLV-derived transfer vector encoding the luciferase reporter protein were transfected using FuGENE6 (Roche, Indianapolis, IN) into 2.5×10^6 293T cells seeded in 10 cm dish 1 day earlier. The medium was replaced with fresh complete DMEM 6 hours after transfection. Supernatants containing the HCVpp were harvested 48 hours later, cleared by passage through 0.45-μM pore-size filters, and used for infection assays. The target Huh-7.5.1 cells were seeded in 12-well plates at a density of 5×10^4 cells per well, incubated overnight at 37°C, and infected with the HCVpp in the presence of $25(OH)D_3$ or ethanol. At 16 hours postinfection,

Address reprint requests to: Takanobu Kato, M.D., Ph.D., Department of Virology II, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan. E-mail: takato@nih.go.jp or Michio Imawari, M.D., Ph.D., Division of Gastroenterology, Department of Medicine, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8666, Japan. E-mail: imawari@med.showa-u.ac.jp.

View this article online at wileyonlinelibrary.com.
DOI 10.1002/hep.25763
Potential conflict of interest: Nothing to report.
Additional Supporting Information may be found in the online version of this article.

medium was replaced with DMEM with 10% fetal bovine serum, and cells were harvested 24 hours later for analysis of luciferase activity.

HCV Subgenomic Replicon Assay. The transient assay of the genotype 2a (JFH-1) subgenomic reporter replicon has been reported.[21] This subgenomic replicon contains the firefly luciferase reporter gene and enables assessment of HCV replication by measuring the luciferase activities in culture cells. Four hours after transfection, the cells in a portion of the plates were harvested as a control for transfection efficiency, and a remaining portion was treated with 25(OH)D_3 or ethanol. The cells were harvested 72 hours after transfection for luciferase measurement. Replication efficiency of HCV in each preparation was calculated as the percent of luciferase activity under the ethanol treatment after normalization by transfection efficiency.

Titration of HCV Infectivity. Culture medium was diluted 10-fold with phosphate-buffered saline and concentrated with Amicon Ultra-15 centrifugal filter units (Millipore, Billerica, MA) to avoid carrying over the test substances. Prepared samples were serially diluted in five-fold increments in complete DMEM and used to infect naïve Huh-7.5.1 cells seeded 24 hours earlier in poly-D-lysine–coated flat-bottom 96-well plates (Corning Inc., Corning, NY) at a density of 1×10^4 cells per well. Three days after infection, HCV-positive cells were detected with mouse monoclonal antibody recognizing the core protein (clone 2H9)[15] and visualized with Alexa Fluor 488 anti-mouse secondary antibody (Invitrogen). The infectivity titer was expressed as focus-forming units per milliliter supernatant (ffu/mL), expressing the mean number of HCV core–positive foci detected at the highest dilutions.[22] The intracellular infectivity and specific intracellular infectivity titer were determined as described.[23]

Isolation of a 25(OH)D_3-Resistant Mutant. In order to obtain a resistant mutant for 25(OH)D_3, the serial passage of JFH-1–transfected cells was performed. HuH-7 cells were electroporated with 3 μg of synthetic HCV RNA, resuspended in 10 mL of complete growth medium, and seeded into a 10-cm dish. After 24 hours, transfected cells were cultured with medium containing 1.0 μM of 25(OH)D_3. HCV titer was monitored by measuring the HCV core Ag, and the resistant mutant virus was harvested and stocked at the peak of the core Ag expression. HCV RNA was extracted from culture medium at this point, and complementary DNA was synthesized and amplified via nested PCR covering almost the entire open reading frame and part of the 5′ untranslated region as described.[23]

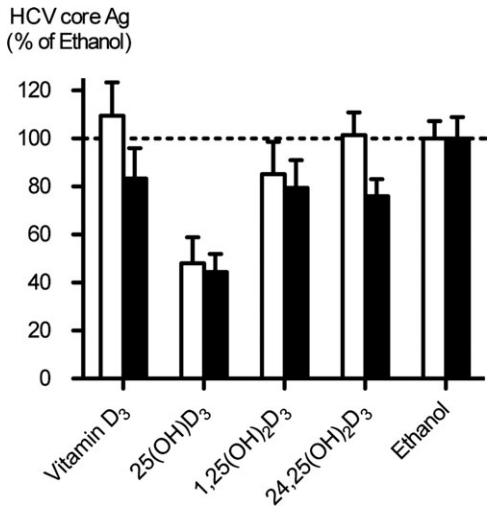

Fig. 1. Anti-HCV effect of vitamin D_3 and its metabolites. HuH-7 cells were pretreated with vitamin D_3, its metabolites, or ethanol (solvent control) and inoculated with HCVcc. HCV production was assessed by measuring the HCV core Ag after a 3-day treatment. HCV core Ag in culture medium and cell lysates are indicated by white and black bars, respectively. Results are expressed as the mean ± SD of the percent of the control (ethanol treatment).

Statistical Analysis. Assays were performed in triplicate. Data from repeated experiments are expressed as the mean ± SD. Statistical analysis was performed using the Student t test, and $P < 0.05$ was considered statistically significant.

Results

Anti-HCV Effect of 25(OH)D_3. To assess the anti-HCV effects of vitamin D_3 and its metabolites—25(OH)D_3, 1,25(OH)$_2$ D_3, and 24,25(OH)$_2D_3$—the HCVcc system was exploited. HuH-7 cells were treated with vitamin D_3 and its metabolites at a concentration of 1.0 μM and were infected with cell culture–adapted HCVcc at a multiplicity of infection of 0.5. Treatment with 25(OH)D_3 markedly reduces HCV core Ag levels in culture medium and cell lysate to 47.97 ± 10.89% and 44.39 ± 7.52% of the control levels, respectively (Fig. 1). Treatment with vitamin D_3 and 1,25(OH)$_2$ D_3 have no remarkable effects. The anti-HCV effect of 25(OH)D_3 on the core Ag is concentration-dependent from 0.125 to 1.0 μM (Fig. 2A). The calculated 50% effective concentration (EC_{50}) of core Ag in culture medium is 0.95 μM. A similar inhibitory effect was also observed for the HCV RNA titer (Fig. 2B). At the higher concentrations of 5 μM, 25(OH)D_3 reduced HCV core Ag levels to 0.4-1.8 % of control (Supporting Fig. 1A). WST-8 assay demonstrated no cytotoxicity in the cells treated with 25(OH)D_3 at a concentration of up

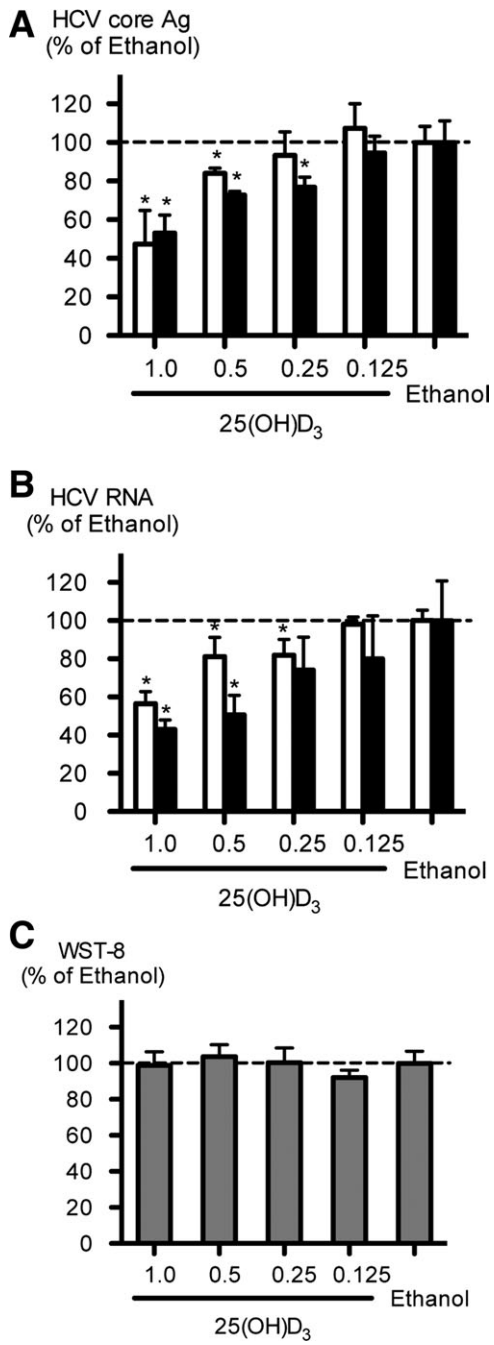

Fig. 2. Dose-dependent anti-HCV effects of 25(OH)D_3. HuH-7 cells were treated with various concentrations of 25(OH)D_3 followed by inoculation with HCVcc. HCV production was assessed by measuring the HCV core Ag. (A, B) HCV core Ag (A) and HCV RNA (B) in culture medium and cell lysates are indicated by white and black bars, respectively. *$P < 0.05$ versus ethanol. (C) Cell viability was assessed via WST-8 assay. Results are expressed as the mean ± SD of the percent of the control.

to 2 μM, and the calculated 50% cytotoxic concentration (CC_{50}) was 7.5 μM (Fig. 2C and Supporting Fig. 1B).

Effects of 25(OH)D_3 in HCV Life Cycle. To evaluate the effects of 25(OH)D_3 on the HCV entry step, Huh-7.5.1 cells were infected with HCVpp harboring envelope glycoproteins of various HCV clones (H77, TH, JFH-1, and J6CF) or vesicular stomatitis virus in the presence of 25(OH)D_3 (1.0, 0.5, and 0.25 μM), or ethanol. Treatment with 25(OH)D_3 has no effects on the relative luciferase activity in the cells infected with HCVpp of any clone, suggesting that 25(OH)D_3 does not affect the HCV entry (Fig. 3A).

To assess the effects of 25(OH)D_3 on HCV replication, we used the HCV subgenomic replicon system. The reporter subgenomic replicon RNA containing luciferase reporter gene was transfected into HuH-7 cells, and the cells were treated with 25(OH)D_3 at a concentration of 0.125 - 1.0 μM or with ethanol. Relative luciferase activities of 25(OH)D_3-treated cells were comparable to those of ethanol-treated cells, indicating no effect on HCV replication (Fig. 3B).

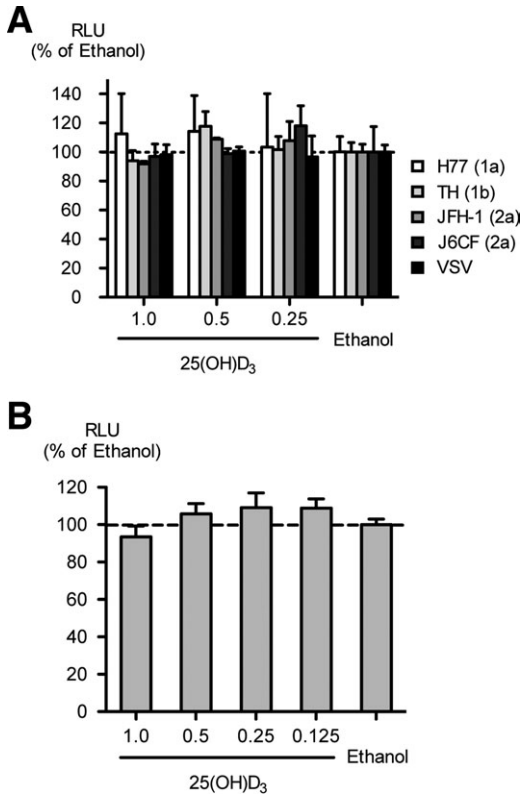

Fig. 3. Effect of 25(OH)D_3 on HCV entry and replication. (A) HCVpp harboring E1 and E2 glycoproteins of various HCV clones and vesicular stomatitis virus G envelope glycoprotein were used to determine the effect of 25(OH)D_3 on the entry step of HCV. Efficiency of HCVpp infection was estimated by measuring the luciferase activity. Results are expressed as the mean ± SD of the percent of the control. (B) HuH-7 cells were transfected with the subgenomic replicon RNAs and treated with various concentrations of 25(OH)D_3. Replication efficiency of the replicon was estimated by measuring the luciferase activity. Results are expressed as the mean ± SD of the percentage of ethanol.

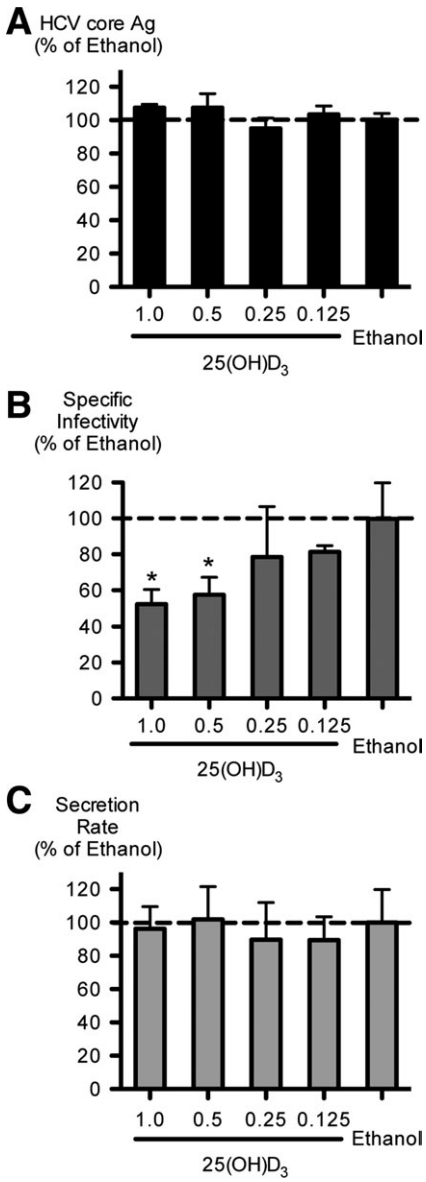

Fig. 4. Effects of 25(OH)D_3 on single-cycle virus production assay. Huh7-25 cells were transfected with JFH-1/wt RNA and treated with various concentrations of 25(OH)D_3. Intracellular HCV core Ag (A), intracellular specific infectivity (B), and the ratio of intra- to extracellular infectivity (secretion rate) (C) were determined. Results are expressed as the mean ± SD. *$P < 0.05$ versus ethanol.

Effects of 25(OH)D_3 in Single-Cycle Virus Production Assay. To further assess whether 25(OH)D_3 affects other steps of the viral life cycle, we used a single-cycle virus production assay with Huh7-25 cells lacking CD81 expression on the cell surface.[14] This cell line can support only replication and infectious virus production upon transfection of HCV genomic RNA but cannot be reinfected by produced HCV, therefore allowing the assessment of a single cycle of infectious viral production.[23] After a 3-day treatment with various concentrations of 25(OH)D_3, extra- and intracellular infectivity and intracellular HCV core Ag were determined. The intracellular levels of HCV core Ag in 25(OH)D_3-treated cells were similar to those of ethanol-treated cells (Fig. 4A and Supporting Table 1), corroborating the observation from the subgenomic replicon experiment. To estimate the efficiency of viral particle assembly, we next determined the intracellular specific infectivity by calculating the ratio of the intracellular infectivity titer over the intracellular HCV core Ag level. Indeed, 25(OH)D_3 at 0.5 and 1.0 μM reduced the intracellular-specific infectivity by approximately half compared with the control ($P < 0.05$) (Fig. 4B and Supporting Table 1). To estimate the infectious virus secretion, we determined the secretion rate by calculating the ratio of the extra- to intracellular infectivity. This analysis revealed no effects of 5(OH)D_3 on the secretion rate at any dose (Fig. 4C and Supporting Table 1).

Enhancement of Anti-HCV Effects of IFN by Supplementation of 25(OH)D_3. Vitamin D supplementation has been shown to improve the efficacy of IFN-based therapy. Therefore, we evaluated the anti-HCV effect of IFN-alpha2b via supplementation of 25(OH)D_3. HuH-7 cells were treated with IFN-alpha2b at concentrations of 1 or 10 IU/mL in combination with 25(OH)D_3 (1.0 μM) or ethanol and were infected with HCVcc. Treatment with 1 IU/mL of IFN-alpha2b reduced HCV core Ag levels in culture medium and cell lysate to 68.66 ± 2.17% and 41.18 ± 1.52% of the control, respectively (Fig. 5). Concomitant treatment with 25(OH)D_3 augments IFN-induced reduction in HCV core Ag levels by approximately two-fold. Similar effects of 25(OH)D_3 are observed with 10 IU/mL IFN-alpha2b.

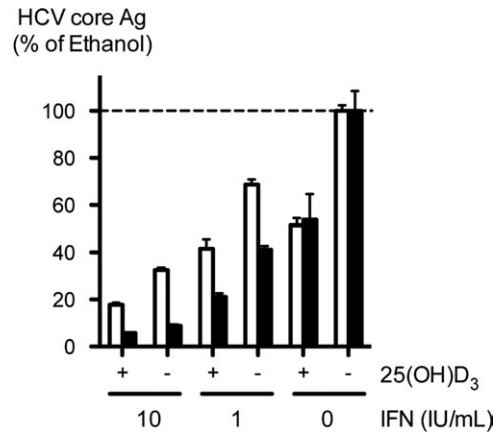

Fig. 5. Enhancement of anti-HCV effects of IFN via supplementation of 25(OH)D_3. HuH-7 cells were treated with IFN at the indicated concentrations in combination with 25(OH)D_3 (1.0 μM) or ethanol, followed by inoculation with HCVcc. HCV production was assessed by measuring the HCV core Ag. HCV core Ag levels in culture medium and cell lysates are indicated by white and black bars, respectively.

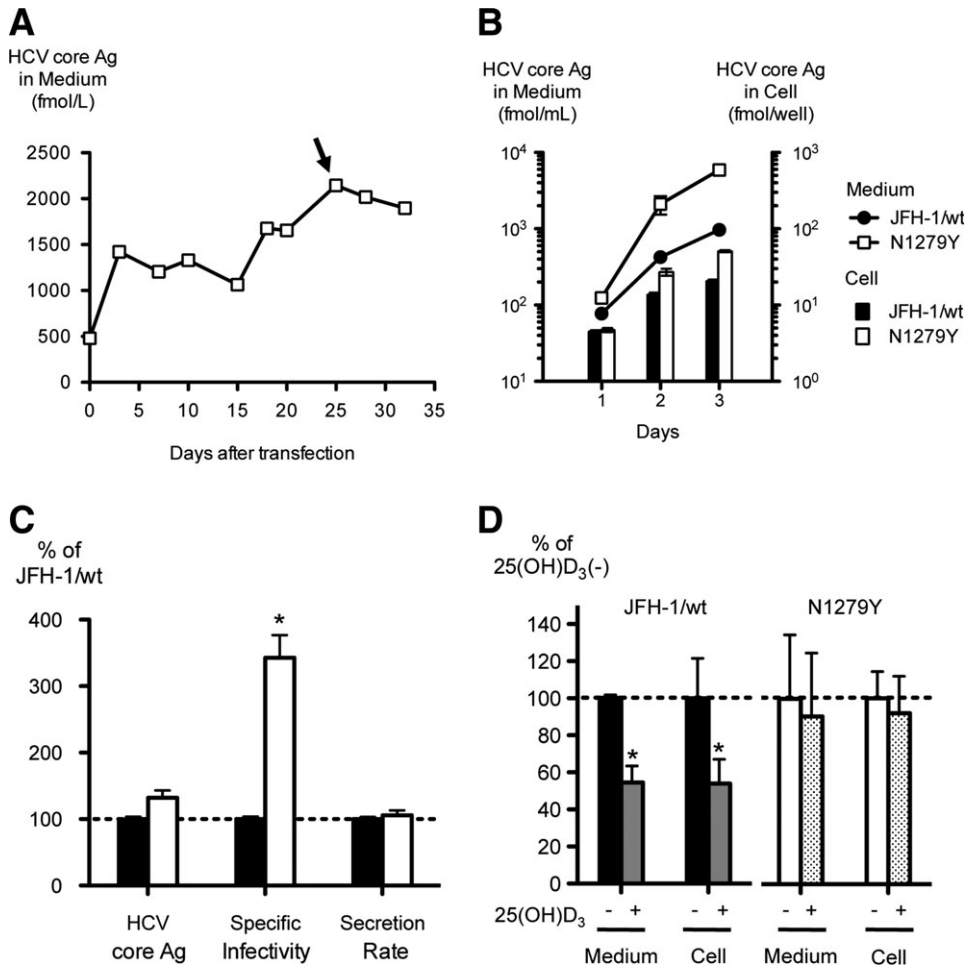

Fig. 6. Emergence of a resistant mutant in long-term culture with $25(OH)D_3$. (A) JFH-1/wt-transfected HuH-7 cells were continuously treated with $25(OH)D_3$ for 32 days to demonstrate the emergence of a resistant mutant. The level of HCV core Ag in culture medium was monitored. The peak point is indicated by an arrow. (B) Comparison of viral production between JFH-1/wt and N1279Y. HCV core Ag in culture medium (lines) and cell lysates (bars) were indicated. Results are expressed as the mean ± SD. (C) Single-cycle virus production assay with JFH-1 and N1279Y. Intracellular HCV core Ag, intracellular specific infectivity, and secretion rate of JFH-1/wt (black bars) and N1279Y (white bars) were depicted. Results are expressed as the mean ± SD of the percentage of JFH-1/wt. $*P < 0.05$ in comparison between N1279Y and wild-type. (D) Anti-HCV effects of $25(OH)D_3$ on JFH-1/wt and N1279Y. Infectivity titer in culture medium and cell lysates were determined. Results are expressed as the mean ± SD of the percentage of ethanol. $*P < 0.05$ versus ethanol.

Effects of 25(OH)D₃ on IFN-Stimulated Gene Induction. Vitamin D has emerged as a key regulator of innate immunity in humans. Type 1 IFN induces many effector molecules known as IFN-stimulated genes (ISGs) with antiviral activities. In order to assess the effects of $25(OH)D_3$ on ISG induction, HuH-7 cells were treated with IFN-alfa2b (10 IU/mL) in the presence or absence of $25(OH)D_3$ (1.0 μM), and the expression of MxA and 2′,5′-oligoadenylate synthetase messenger RNAs were determined by quantitative real-time PCR. The treatment with $25(OH)D_3$ does not affect either basal expression or IFN-mediated induction of MxA and 2′,5′-oligoadenylate synthetase (Supporting Fig. 2).

Isolation of 25(OH)D₃ Resistant Mutant. Next, we determined whether a long-term treatment with $25(OH)D_3$ leads to generation of a $25(OH)D_3$-resistant mutant in JFH-1 transfected cells. During a continuous treatment with $25(OH)D_3$ (1.0 μM) over a period of 32 days, the level of HCV core Ag in culture medium indeed increases gradually (Fig. 6A), suggesting an emergence of a mutant. Therefore, we harvested the culture medium at peak point at day 25, isolated the HCV RNA from the medium, and determined the sequence of entire ORF. Comparative sequencing of the JFH-1 has detected only one amino acid substitution of N to Y at amino acid 1279 (N1279Y) in nonstructural region 3 (NS3). To test the characteristics of this amino acid substitution, we generated a full-genome JFH-1 construct with this substitution (N1279Y) and compared virus replication and production with the wild-type JFH-1 (JFH-1/wt). After transfection of RNA of JFH-1/wt or N1279Y into Huh-7.5.1 cells, HCV core Ag in culture medium and cell lysate were quantified. Both intra- and extracellular levels of HCV core Ag from the N1279Y-transfected cells were significantly higher than those of JFH-1/wt transfected cells ($P < 0.05$) (Fig. 6B). To analyze the effects of this substitution on different stages of the virus life cycle, we performed a single-cycle virus production assay. We compared intracellular HCV core Ag, intracellular specific infectivity, and secretion rate via transfection of JFH-1/wt and N1279Y RNAs into Huh7-25 cells. Our results show that the intracellular specific infectivity was 3.4-fold higher in N1279Y-transfected cells than in JFH-1/wt–transfected cells.

On the other hand, the intracellular core Ag level and secretion rate were not affected by this substitution (Fig. 6C and Supporting Table 2). To test whether this amino acid substitution confers the resistance to the inhibitory effect of $25(OH)D_3$, we compared the effect on infectious virus production. As predicted, treatment with $25(OH)D_3$ at a concentration of 1.0 μM reduced intra- and extracellular infectivity titer in JFH-1/wt–transfected cells to 54.5 $\pm$ 9.0 and 59.1 $\pm$ 14.4% of the control, respectively ($P < 0.05$). However, in N1279Y-transfected cells, these effects were completely abrogated (Fig. 6D), suggesting that the N1279Y mutation was responsible for the $25(OH)D_3$ resistance.

Anti-HCV Effects of 25(OH)D$_3$ in Multiple HCV Strains Other than JFH-1. To assess the strain specificity, we also examined the anti-HCV effect of $25(OH)D_3$ using other HCV strains, J6/JFH-1, H77S.2, and JFH-1 with V2440L mutation (JFH-1/V2440L). Treatment with $25(OH)D_3$ reduced intra- and extracellular infectivity titers of these strains to 25.2-48.2% of the ethanol control (Supporting Fig. 3). Thus, these strains are also susceptible to $25(OH)D_3$.

Production of 1,25(OH)$_2$D$_3$ in Cell Culture. Finally, we tested the possibility that the anti-HCV effect of $25(OH)D_3$ is mediated by $1,25(OH)_2D_3$ generated from $25(OH)D_3$ in the cells. HuH-7 cells were treated with $25(OH)D_3$ at various concentrations, and production of $1,25(OH)_2D_3$ was measured. After treatment of $25(OH)D_3$ at 5.0 and 2.0 μM, the concentrations of $1,25(OH)_2D_3$ detected in the culture medium were 365.62 $\pm$ 30.01 and 104.49 $\pm$ 11.08 pM, respectively, and are slightly higher than that with ethanol treatment (Supporting Fig. 4A). After treatment of $25(OH)D_3$ at 1.0 and 0.5 μM, the $1,25(OH)_2D_3$ concentrations are similar to that of ethanol treatment. We then evaluated whether the concentrations of $1,25(OH)_2D_3$ ranging from 0.01 to 1.0 μM have an anti-HCV effect on HCVcc-infected cells. This study revealed no anti-HCV effect at any concentration studied, although an anti-HCV effect of $25(OH)D_3$ is reproducible (Supporting Fig. 4B).

Discussion

Vitamin D is reported to have regulatory roles in infectious diseases[12,24] and to act as an immune modulator in both innate and adaptive immune pathways.[25,26] In patients with chronic hepatitis C, low serum levels of vitamin D are associated with severe fibrosis, and poor responsiveness to IFN-based therapy has been reported.[27] Furthermore, vitamin D supplementation significantly improves the SVR rate of combination IFN and ribavirin therapy in patients with hepatitis C.[5,6] These studies postulated that $1,25(OH)_2D$, the active metabolite of 25(OH)D, is responsible for the effects. However, our current study using the culture model, identifies $25(OH)D_3$ but not $1,25(OH)_2D_3$ as an effective anti-HCV metabolite with the ability to suppress infectious virus production (Figs. 1 and 2). By using HCVpp and a subgenomic replicon system, we found that $25(OH)D_3$ did not influence the steps of HCV entry and replication (Fig. 3) but rather selectively inhibited the virus assembly step (Fig. 4). We believe that this finding offers a novel mechanistic insight into the therapeutic efficacy of supplemental vitamin D observed in HCV patients.

Detailed mechanisms of the anti-HCV effect of $25(OH)D_3$ are still elusive. The biological activity of vitamin D is mainly attributed to $1,25(OH)_2D$, the most active form of vitamin D, and 25(OH)D has only been regarded as a pro-hormone with no ascribed direct biological functions. However, a recent study demonstrates gene regulatory effects of $25(OH)D_3$ in a manner dependent on vitamin D receptor and its synergism with $1,25(OH)_2D_3$.[28] It is still unclear whether 25(OH)D has direct targets or biological functions different from those of $1,25(OH)_2D$. Our study demonstrates in HCVcc-infected cells a clear difference in the effect on HCV viral assembly rendered by $25(OH)D_3$ versus $1,25(OH)_2D_3$, with only the former having an inhibitory effect (Fig. 1, Supporting Fig. 4); this finding suggests that the common signaling pathway cannot underlie this discrete action. The treatment with $25(OH)D_3$ may induce specific host factors involved in inhibition of infectious HCV production. To this end, we evaluated ISG induction by $25(OH)D_3$ with or without IFN treatment, but no effects were observed (Supporting Fig. 2), suggesting that involvement of ISGs is unlikely in the observed effect of $25(OH)D_3$.

The anti-HCV effect of $25(OH)D_3$ is limited in its potency; infectious virus production is reduced only to half of controls (Figs. 1 and 2). This reduction may not be sufficient when it is used as a sole anti-HCV agent. Moreover, in our long-term culture experiment with $25(OH)D_3$, the JFH-1–resistant mutant emerged (Fig. 6). We cloned and sequenced this mutant to demonstrate that the N1279Y substitution in NS3 is responsible for the resistance (Fig. 6D), although this amino acid polymorphism is not observed among deposited strains in the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp/). NS3 is known to code

protease and helicase, and the detected substitution is in the helicase domain. This substitution is associated with a marked enhancement of assembly efficiency of infectious virus particles (Fig. 6C). As the involvement of the NS3 helicase domain in virion morphogenesis is reported,[29] the observed suppressive effect of $25(OH)D_3$ may be associated with this specific function of NS3. Collectively, our findings suggest that the treatment of hepatitis C with $25(OH)D_3$ alone may not be recommended because of a limited antiviral effect and emergence of the resistant mutant. However, if it is combined with compounds that inhibit the HCV replication such as IFN or protease inhibitors, it should optimize the antiviral effect while minimizing the genesis of the mutant. Indeed, we confirm the enhancement of anti-HCV effect of IFN by supplementation of $25(OH)D_3$ (Fig. 5).

The effective concentrations of $25(OH)D_3$ shown in our study may seem too high. We used 1 μM of $25(OH)D_3$ to observe the sufficient reduction of infectious virus production (Fig. 2). This concentration is almost 10-fold higher than the peripheral concentration in normal subjects. However, the actual concentration of 25(OH)D may be much higher in the liver where this metabolite is primarily produced. Because HCV replicates in the same hepatocellular site, 25(OH)D may be sufficiently concentrated to render an immediate anti-HCV effect. On the other hand, low 25(OH)D levels are reported in patients with cirrhosis or severe hepatic dysfunction.[30-33] A depressed activity of 25-hydroxylase in damaged livers in these patients may be responsible for this observation. Therefore, 25(OH)D, not vitamin D, should be a better antiviral agent in such patients as a supplement for IFN and ribavirin therapy.

In a recent report, Gal-Tanamy et al.[34] describe the anti-HCV effects of vitamin D. In this study, treatment with vitamin D_3 or $1,25(OH)_2D_3$ reduced infectious virus production by Huh-7.5 cells infected with HCV H77-JFH-1 intergenotypic chimeric virus. The effects are ascribed to enhancement of IFN-beta expression and ISG induction by $1,25(OH)2D_3$. In our experiments, we did not observe such anti-HCV effects of vitamin D_3 or $1,25(OH)_2D_3$ at the concentrations similar to those used by Gal-Tanamy et al. This discrepancy might be related to the different systems used for analyses. The anti-HCV activities of vitamin D and its metabolites may be different among HCV strains and cell lines. Further studies will be needed to resolve this discrepancy.

In conclusion, $25(OH)D_3$ is identified as a novel anti-HCV agent that selectively targets the infectious viral particle assembly step. This observation provides the basis for the improvement of efficacy of anti-HCV treatment by supplementation of vitamin D to IFN and ribavirin therapy. Our study also provides the possibility that the supplementation of 25(OH)D would be indicated in patients with compromised hepatic functions. Clinical studies are required to evaluate a possible therapeutic use of 25(OH)D.

Acknowledgment: We are grateful to Francis V. Chisari (Scripps Research Institute, La Jolla, CA) for Huh-7.5.1 cells and to Stanley M. Lemon (University of North Carolina at Chapel Hill, Chapel Hill, NC) for pH77S.2 the plasmid for H77S.2 (replication competent genotype 1a strain).

References

1. Liang TJ, Rehermann B, Seeff LB, Hoofnagle JH. Pathogenesis, natural history, treatment, and prevention of hepatitis C. Ann Intern Med 2000;132:296-305.
2. Seeff LB, Hoofnagle JH. National Institutes of Health Consensus Development Conference: management of hepatitis C: 2002. Hepatology 2002;36:S1-S2.
3. Feld JJ, Hoofnagle JH. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. Nature 2005;436:967-972.
4. Pawlotsky JM. Therapy of hepatitis C: from empiricism to eradication. Hepatology 2006;43:S207-S220.
5. Bitetto D, Fabris C, Fornasiere E, Pipan C, Fumolo E, Cussigh A, et al. Vitamin D supplementation improves response to antiviral treatment for recurrent hepatitis C. Transpl Int 2011;24:43-50.
6. Abu-Mouch S, Fireman Z, Jarchovsky J, Zeina AR, Assy N. Vitamin D supplementation improves sustained virologic response in chronic hepatitis C (genotype 1)-naive patients. World J Gastroenterol 2011; 17:5184-5190.
7. Imawari M, Kida K, Goodman DS. The transport of vitamin D and its 25-hydroxy metabolite in human plasma. Isolation and partial characterization of vitamin D and 25-hydroxyvitamin D binding protein. J Clin Invest 1976;58:514-523.
8. Garland CF, Garland FC, Gorham ED, Lipkin M, Newmark H, Mohr SB, et al. The role of vitamin D in cancer prevention. Am J Public Health 2006;96:252-261.
9. VanAmerongen BM, Dijkstra CD, Lips P, Polman CH. Multiple sclerosis and vitamin D: an update. Eur J Clin Nutr 2004;58:1095-1109.
10. Zittermann A. Vitamin D and disease prevention with special reference to cardiovascular disease. Prog Biophys Mol Biol 2006;92:39-48.
11. Davies PD, Brown RC, Woodhead JS. Serum concentrations of vitamin D metabolites in untreated tuberculosis. Thorax 1985;40:187-190.
12. Muhe L, Lulseged S, Mason KE, Simoes EA. Case-control study of the role of nutritional rickets in the risk of developing pneumonia in Ethiopian children. Lancet 1997;349:1801-1804.
13. Chiu KC, Chu A, Go VLW, Saad MF. Hypovitaminosis D is associated with insulin resistance and β cell dysfunction. Am J Clin Nutr 2004; 79:820-825.
14. Akazawa D, Date T, Morikawa K, Murayama A, Miyamoto M, Kaga M, et al. CD81 expression is important for the permissiveness of Huh7 cell clones for heterogeneous hepatitis C virus infection. J Virol 2007; 81:5036-5045.
15. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791-796.

16. Takeuchi T, Katsume A, Tanaka T, Abe A, Inoue K, Tsukiyama-Kohara K, et al. Real-time detection system for quantification of hepatitis C virus genome. Gastroenterology 1999;116:636-642.
17. Aoyagi K, Ohue C, Iida K, Kimura T, Tanaka E, Kiyosawa K, et al. Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen. J Clin Microbiol 1999;37:1802-1808.
18. Ishiyama M, Miyazono Y, Sasamoto K, Ohkura Y, Ueno K. A highly water-soluble disulfonated tetrazolium salt as a chromogenic indicator for NADH as well as cell viability. Talanta 1997;44:1299-1305.
19. Bartosch B, Dubuisson J, Cosset FL. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. J Exp Med 2003;197:633-642.
20. Bartosch B, Vitelli A, Granier C, Goujon C, Dubuisson J, Pascale S, et al. Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. J Biol Chem 2003;278:41624-41630.
21. Kato T, Date T, Miyamoto M, Sugiyama M, Tanaka Y, Orito E, et al. Detection of anti-hepatitis C virus effects of interferon and ribavirin by a sensitive replicon system. J Clin Microbiol 2005;43:5679-5684.
22. Kato T, Date T, Murayama A, Morikawa K, Akazawa D, Wakita T. Cell culture and infection system for hepatitis C virus. Nat Protoc 2006;1:2334-2339.
23. Kato T, Choi Y, Elmowalid G, Sapp RK, Barth H, Furusaka A, et al. Hepatitis C virus JFH-1 strain infection in chimpanzees is associated with low pathogenicity and emergence of an adaptive mutation. HEPATOLOGY 2008;48:732-740.
24. Yuk JM, Shin DM, Lee HM, Yang CS, Jin HS, Kim KK, et al. Vitamin D3 induces autophagy in human monocytes/macrophages via cathelicidin. Cell Host Microbe 2009;6:231-243.
25. Lin R, White JH. The pleiotropic actions of vitamin D. Bioessays 2004;26:21-28.
26. Liu PT, Modlin RL. Human macrophage host defense against Mycobacterium tuberculosis. Curr Opin Immunol 2008;20:371-376.
27. Petta S, Camma C, Scazzone C, Tripodo C, Di Marco V, Bono A, et al. Low vitamin D serum level is related to severe fibrosis and low responsiveness to interferon-based therapy in genotype 1 chronic hepatitis C. HEPATOLOGY 2010;51:1158-1167.
28. Lou YR, Molnar F, Perakyla M, Qiao S, Kalueff AV, St-Arnaud R, et al. 25-Hydroxyvitamin D(3) is an agonistic vitamin D receptor ligand. J Steroid Biochem Mol Biol 2010;118:162-170.
29. Jones DM, Atoom AM, Zhang X, Kottilil S, Russell RS. A genetic interaction between the core and NS3 proteins of hepatitis C virus is essential for production of infectious virus. J Virol 2011;85:12351-12361.
30. Monegal A, Navasa M, Guanabens N, Peris P, Pons F, Martinez de Osaba MJ, et al. Osteoporosis and bone mineral metabolism disorders in cirrhotic patients referred for orthotopic liver transplantation. Calcif Tissue Int 1997;60:148-154.
31. Hofmann WP, Kronenberger B, Bojunga J, Stamm B, Herrmann E, Bucker A, et al. Prospective study of bone mineral density and metabolism in patients with chronic hepatitis C during pegylated interferon alpha and ribavirin therapy. J Viral Hepat 2008;15:790-796.
32. Imawari M, Akanuma Y, Itakura H, Muto Y, Kosaka K, Goodman DS. The effects of diseases of the liver on serum 25-hydroxyvitamin D and on the serum binding protein for vitamin D and its metabolites. J Lab Clin Med 1979;93:171-180.
33. Masuda S, Okano T, Osawa K, Shinjo M, Suematsu T, Kobayashi T. Concentrations of vitamin D-binding protein and vitamin D metabolites in plasma of patients with liver cirrhosis. J Nutr Sci Vitaminol (Tokyo) 1989;35:225-234.
34. Gal-Tanamy M, Bachmetov L, Ravid A, Koren R, Erman A, Tur-Kaspa R, et al. Vitamin-D: an innate antiviral agent suppressing Hepatitis C virus in human hepatocytes. HEPATOLOGY 2011;54:1570-1579.