small assay volumes are required for maximum efficiency, in order to minimize expense, space requirements, reagent consumption, and experimental time. The advancement of HTS technologies has made it possible to execute cell-based HTS with 384-well and even 1536-well plates. Use of automated methods for plate filling, compound transfer, and plate reading further maximizes efficiency. With the assistance of sophisticated software tools, screening data can be rapidly analyzed in an automated fashion.[5-7]

In addition to an efficient screening platform, large, diverse libraries of small molecule pertubagens appropriately formatted for this platform are required. The known bioactives library, a collection of 2568 known and proven bioactive molecules, including FDA-approved drugs, was the library screened in this report. Also available were commercially available combinatorial chemistry libraries and several diversity-oriented synthesis (DOS) libraries. One of these DOS libraries, Project Kornberg (a library of over 8000 DOS molecules that links chemists from multiple research groups throughout the US), was also screened using the methodology developed in this article. These results are the subject of a separate report.

Finally, a cell line carrying an accurate and reliable reporter is needed to efficiently screen large small-molecule libraries. To this end, the selection and optimization of the most appropriate HCV replicon cell line is very important. To successfully perform high-density HTS using 384-well plates, several characteristics of an HCV replicon cell line must be taken into account, such as RNA replication levels carrying an easy read-out reporter gene and host cell growth characteristics.

We selected the subgenomic Huh7/Rep-Feo replicon cell line because of its robust level of replication.[8] The level of HCV replication appears to be very high in this replicon cell line, although the precise comparison of replication levels between various replicon cell lines is difficult because of differing conditions in different laboratories.[16-19] We chose a replicon bearing the firefly luciferase gene because it can be easily measured, is very sensitive, and has a suitably long half-life.[8,20] Indeed, we obtained a high S/B ratio (>100) in the 384-well plate format, ensuring the suitability of this system for high-density HTS assays.

To facilitate detection of HCV replication, several replicon cell lines that carry reporter genes, such as beta-lactamase,[21] luciferase,[8,9,20] and secreted alkaline phosphatase (SEAP),[22,23] have also been developed. In fact, a cell-based HTS assay using 384-well plates has been developed with an HCV replicon bearing a beta-lactamase reporter gene.[24] However, this replicon model has several disadvantages. There is a relatively low signal to background. Moreover, additional processing is required to suppress the high background signal. Finally, because the cell line was transiently transfected, it requires extensive preparation prior to screening. *Renilla* luciferase has also been used as a reporter gene, but is not an ideal choice because of its very short signal half-life. Moreover, aspiration and lysis processing steps must be carried out prior to signal detection.[9] On the other hand, replicon cell models bearing a SEAP reporter do not require aspiration and lysis steps.[22,23] These systems, however, either require another viral protein, such as tat, to express the reporter protein or require the action of NS3/4A-specific protease activity.[22,23] Replicons bearing firefly luciferase reporter genes, therefore, appear to be better suited for use in HTS assays.

Many small molecules are cytotoxic, and hepatocyte replicon cell lines are highly sensitive to cytotoxic or cytostatic agents.[8,20] Cytotoxic effects can be mistaken for antiviral activity by decreasing luciferase signal merely by decreasing cell viability and not by decreasing HCV RNA replication, leading to false-positive results. In fact, when we confined our analysis to the reporter gene assay alone, our hit rates were very high, primarily due to false-positive results from cytotoxic compounds. We therefore perform both the reporter gene assay and a cell viability assay in parallel in our primary HTS, a step we view as essential. This counter screen was executed in order to minimize confounding from increased or decreased luciferase signal due to increased or decreased cell viability, respectively.

To verify hit compounds identified from primary screening, an appropriate secondary assay must be performed. We used the subgenomic Huh7/Rep-Feo replicon cell line in our primary screen because of its simplicity, rapidity, robustness, and reproducibility. Its disadvantages include its lack of structural proteins, its specific adaptive mutation, and its firefly luciferase reporter gene. By using a subgenomic replicon, the influence of structural proteins on the antiviral activity of compounds cannot be probed. Furthermore, a cell culture adaptive mutation may affect the biochemical properties of a protein by altering its sensitivity to an antiviral drug.[3,4] Compounds that specifically inhibit firefly luciferase activity can be identified as false-positive antiviral hits. For instance, pifithrin, an antiviral hit in our primary screen, inhibits firefly luciferase, but not *Renilla* luciferase.[25] In the secondary screen, we validated primary hits using a full-length OR6 replicon,[9] thereby ensuring validation in a more authentic viral polyprotein context. This full-length replicon also possesses a cell culture-adaptive mutation and a reporter gene distinct from those found in the subgenomic Huh7/Rep-Feo replicon, thereby minimizing confounding from those factors.

One of the limitations of high-throughput screening is that the generation of adequate dose-response curves for each of the compounds tested in a large primary screen would overwhelm the capacity of even the best screening technology platform by the sheer number of data points generated. Although screening at a single fixed concen-

tration per compound may lead to missing potential hits, it is much more efficient generating the dose-response data only for those compounds identified as hits. We conducted secondary validation screens to generate adequate dose-response curves for our hit compounds. We also performed cell viability assays in our secondary screens to minimize confounding from cytotoxicity.

Corticosteroids have been reported to promote HCV replication. It is thought that they exert their effects via host cellular immunosuppression.[13] However, our results demonstrate the direct proviral effect of corticosteroids in both a subgenomic and a full-length HCV replicon system. These data suggest that corticosteroids are capable of promoting HCV replication independent of their host immunosuppressive activity. Further study of the direct regulation of HCV replication by corticosteroids is warranted.

Strikingly, the statins were found to exhibit powerful antiviral effects. Lovastatin has been shown in an independent model to decrease HCV replication.[12] These compounds are inhibitors of HMG-CoA reductase and shut down cholesterol biosynthesis by preventing the formation of mevalonate from 3-hydroxy-3-methyl-glutaryl CoA. In addition to lowering intracellular levels of sterols, statins also reduce levels of isoprenoids, which are derived from mevalonate. Isoprenoids such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate serve as lipid attachments for a variety of intracellular signaling molecules. It has been reported that inhibition of geranylgeranylation, rather than the synthesis of cholesterol itself, is responsible for the inhibition of HCV RNA replication.[12,26] All statins except pravastatin showed good dose response in the secondary assays, with IC_{50} values from 1-10 μM. The HMG-CoA reductase inhibitors therefore appear to demonstrate potential as antiviral agents.

Recently, Ikeda et al reported anti-HCV profiles of 5 different statins using the OR6 replicon system. They found that fluvastatin exhibited the strongest anti-HCV activity, whereas atorvastatin, simvastatin, and lovastatin had moderate-to-mild anti-HCV activity. Pravastatin did not have significant anti-HCV activity.[27] However, our results show that the anti-HCV activity of the statins in order of decreasing potency was simvastatin, fluvastatin, atorvastatin, mevastatin, lovastatin, and pravastatin. It is likely that the IC_{50} of simvastatin, 1.6 μM, was higher than Ikeda's because our data were obtained at only 48 hours after treatment, as opposed to 72 hours in the Ikeda study. Pravastatin also had anti-HCV activity, although it was very weak (IC_{50}: 238.5 μM, data not shown). Furthermore, as with the study of Ikeda, the anti-HCV effect of the statins, including that of pravastatin, was rescued by addition of geranylgeraniol (data not shown). Because the HCV genome does not encode a geranylgeranylated protein, we hypothesize that a host geranylgeranylated protein must play an important role in HCV replication and that inhibition of the geranylgeranylation of this protein represents a potential strategy for blocking HCV replication. Thus, for statins to exert their anti-HCV effects, they must deplete mevalonate sufficiently to lower the cellular pools of geranylgeranyl pyrophosphates in the replicon cell lines. We speculate that this indirect mechanism of action explains an anti-HCV IC_{50} for statins in the micromolar range, in contrast to their IC_{50} for HMG-CoA reductase in the nanomolar range.[12,28] Potential reasons for pravastatin's observed weaker activity include its higher hydrophilicity compared to the other statins and its lack of metabolism by the cytochrome P450 system.[29]

Although we verified several hit compounds, such as the corticosteroids and the HMG-CoA reductase inhibitors, our validation assays revealed some false positives from our primary screening. Tetrandrine and MY-5445 were antiviral hits in the primary screen that were found to be false positives with significant cytotoxicity in secondary, but not primary, screening. This phenomenon was also observed for lovastatin. These false-positive results may be due in part to the lack of HCV structural proteins in our primary screening system. For example, the expression of HCV core protein has been reported to affect Fas-mediated apoptosis depending on environmental conditions.[30] Thus, it is possible that HCV core, in the presence of small molecules, could lead to apoptosis and cytotoxicity. These findings demonstrate that validation of primary screening results with subgenomic replicon systems should be performed with full-length replicon systems in order to totally assess the potential interactions of these compounds in the authentic viral protein context.

Although our cell-based HTS system represents an effective screening method for identification of potential antiviral agents, there are potential limitations to use of the Huh7/Rep-Feo replicon cell line, which is based on a subgenomic, not full-length, genotype 1b clone. By using the full-length OR6 replicon cell line for our confirmatory secondary assays, we have addressed one particular limitation. With regard to genotype, recent description of a bona fide infectious HCV cell line propagating genotype 2 virus[31,32] will permit assessment of the generalizability of the inhibitory action of these agents against other HCV genotypes. To date, the vast majority of HCV replicons have been developed in Huh7 cells, which appear to be uniquely permissive for these studies. With the eventual development of successful replication models in other hepatocyte cell lines, further testing of these compounds in such lines will also be of interest.

In summary, we have developed a rapid, reliable, reproducible, and validated cell-based HTS method to identify positive and negative regulators of HCV replication. This method can be used not only to identify putative antiviral agents but also cellular regulators of replication. The compounds identified by these screens can be used not

only for medicinal effect but also as a productive approach to the characterization of key viral-host interactions critical to viral replication.

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Received July 10, 2006. Accepted October 5, 2006.

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Supported by NIH NS050854-01 (R.T.C.), the GlaxoSmithKline Research Fund of the Korean Association for The Study of The Liver (S.S.K.), and NIH 5T32DK07191-31 (L.F.P.).

The authors thank the National Cancer Institute and the Initiative for Chemical Genetics, who provided support for this publication, and the Chemical Biology Platform of the Broad Institute of Harvard and MIT for their assistance in this work. The authors also thank Dr Nobuyuki Kato (Okayama University) for his kind gift of the OR6 replicon cell line.

J Pharmacol Sci **105**, 145 – 150 (2007)

Journal of Pharmacological Sciences
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Forum Minireview

Life Style-Related Diseases of the Digestive System: Cell Culture System for the Screening of Anti-Hepatitis C Virus (HCV) Reagents: Suppression of HCV Replication by Statins and Synergistic Action With Interferon

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Received July 10, 2007; Accepted September 10, 2007

Abstract. Hepatitis C virus (HCV) infection causes chronic hepatitis and leads to liver fibrosis and hepatocellular carcinoma. Pegylated-interferon and ribavirin is the current standard therapy for chronic hepatitis C. However, the therapy is only effective in 50% of the patients. To overcome this problem, we recently developed the HCV cell culture system (OR6 system) for the screening of anti-HCV reagents. In this OR6 system, the luciferase gene was introduced into the upstream portion of the HCV genome to facilitate the monitoring of HCV RNA replication. Recently lipid metabolism is reported to be involved in HCV RNA replication. Cholesterol and sphingolipid are the major components in lipid rafts, which seem to be the scaffold for HCV RNA replication. Statins inhibit cholesterol biosynthesis and also have the pleiotropic effects by the inhibition of prenylation. We demonstrated different anti-HCV effects of statins (atorvastatin, simvastatin, fluvastatin, lovastatin, and pitavastatin) using the OR6 system. Surprisingly, in contrast to the other statins, pravastatin exhibited no anti-HCV effect. Furthermore, statins enhanced the anti-HCV effect of interferon in combination. Statins may be a promising candidate for the adjuvant in interferon therapy and may improve the efficiency of the current interferon and ribavirin therapy.

Keywords: life style-related disease, hepatitis C virus (HCV), statin, interferon, cell culture system

Introduction

Approximately 170 million people worldwide are infected with the hepatitis C virus (HCV). HCV infection causes chronic hepatitis C (CH-C) and leads to liver-related death by liver cirrhosis and/or hepatocellular carcinoma. To prevent the progress of fatal liver disease after HCV infection, the elimination of the virus seems to be the most effective strategy. However, the current pegylated-interferon (PEG-IFN) and ribavirin therapy was only effective in 50% of the patients (1). Therefore, the development of more effective anti-HCV reagents is an urgent concern. When HCV replicates in hepatocytes, some of the cellular factors are essential for HCV RNA replication. These cellular factors are the targets for antiviral as well as viral proteins such as NS3 protease or NS5B RNA-dependent RNA polymerase. Inhibition of cellular factors may cause side effects by the inhibition of their primary roles. However, one of the advantages of this strategy is that it could overcome the viral mutation leading to the resistance to the reagent against the viral proteins. Lipid metabolism is one of the candidates in the context of this strategy. To explore the best partner of IFN, we examined different six statins, which are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, using our recently developed OR6 system (2). In the OR6 system, genome-length HCV RNAs (HCV-O strain of genotype 1b) replicate efficiently and the HCV RNA level can be monitored by luciferase activities (3, 4). Statins exhibited various anti-HCV activities except for pravastatin that was not active against HCV (2). We also

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Published online in J-STAGE: October 6, 2007
doi: 10.1254/jphs.FM0070050

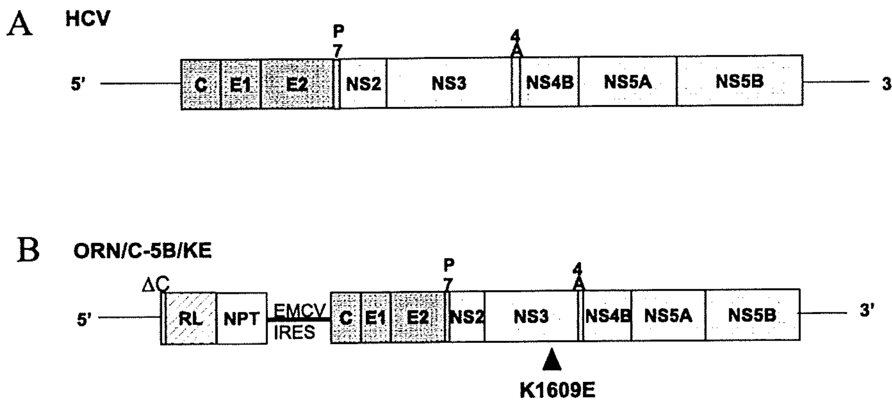

Fig. 1. HCV RNA with reporter gene. Schematic gene organization of genome-length HCV RNA. A: The authentic HCV RNA was composed of the N-terminal part of the structural region and C-terminal part of the nonstructural region. B: The genome-length HCV RNA with reporter gene was constructed based on the authentic HCV RNA. EMCV IRES was introduced for the translation of HCV proteins. Renilla luciferase was expressed as a fusion protein with NPT. The position of the adaptive mutation, K1609E, is indicated by a black triangle.

investigated whether or not statins could enhance the inhibitory effect of IFN on HCV RNA replication. In this review, we would like to summarize our recent findings and the literature regarding lipid metabolism as the target of anti-HCV with a focus on statins.

Cell culture system for HCV RNA replication

Cell culture systems for HCV have been developed since the first breakthrough of the establishment of the subgenomic replicon by Lohmann et al. (5). The replicon system has provided the information concerning the mechanism of the replication machinery of HCV and has revealed the cellular factors essential for HCV RNA replication. After the development of the subgenomic replicon, genome-length HCV RNA replication systems using different HCV strains (H, N, Con1, and O) were developed by several groups since the subgenomic replicon did not possess the structural region in the genome (4, 6 – 8). For the screening of anti-HCV reagents, the replicon system has also been improved by the introduction of reporter genes (9). The introduction of the reporter gene into the HCV genome facilitated the monitoring of HCV RNA replication. For this purpose, we developed a cell culture system (OR6 system) in which genome-length HCV RNA containing renilla luciferase (RL) replicate efficiently under the selection by G418 (4). As shown in Fig. 1, RL, neomycin phosphotransferase (NPT), and encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES) genes were introduced between the 5' untranslated region and Core (C) of HCV. This genome-length HCV RNA robustly replicated in the hepatoma cell line HuH-7 after the electroporation and one of the colonies designated OR6 was selected by G418 and used for the studies including determining the anti-HCV effect of statins. A recent milestone was the development of an HCV infection system using a genotype 2a HCV strain, JFH-1 (10 – 12). This system could reconstruct the HCV life cycle in cell culture. The future issue of the cell culture system is the development of a robust genotype 1 HCV virus production system because the efficiency of PEG-IFN and ribavirin therapy in patients with genotype 1 HCV remained lower than that in patients with genotype 2 HCV: the sustained virological responses were approximately 50% versus 80% – 90%, respectively (13). More recently, pioneering studies have been reported by several groups using genotype 1 HCV strains for virus production (14, 15). However, the genotype 1 HCV virus production systems could not allow re-infection with the supernatant from the HCV-infected cells. These ongoing studies will lead to the development of a robust genotype 1 HCV infection system like genotype 2a HCV in the near future.

HCV and lipid metabolism

Lipid metabolism is involved in the life cycle of many viruses. The resulting metabolites work as physiologically active molecules such as eicosanoids and so on, and some of them are incorporated into the lipid raft membrane. A lipid raft is distinct from other lipid membranes. It is enriched in cholesterols and sphingolipids and is detergent-resistant. Lipid rafts play an important role in virus entry, replication, and assembly. HCV also forms a replication complex on the lipid raft membrane structure (16). Therefore, the depletion of the cholesterol and sphingolipid from the lipid raft leads to the inhibition of HCV RNA replication. Aizaki et al. (17) reported that lovastatin inhibited HCV RNA replication in HCV replicon-harboring cells. Statins are inhibitors for HMG-CoA reductase in the cholesterol biosynthesis pathway (Fig. 2). Statins also possess the cholesterol-independent action (pleiotropic effect) (18). Many of these pleiotropic effects are mediated by the isoprenoid. Farnesyl pyrophosphate (FPP) and gera-

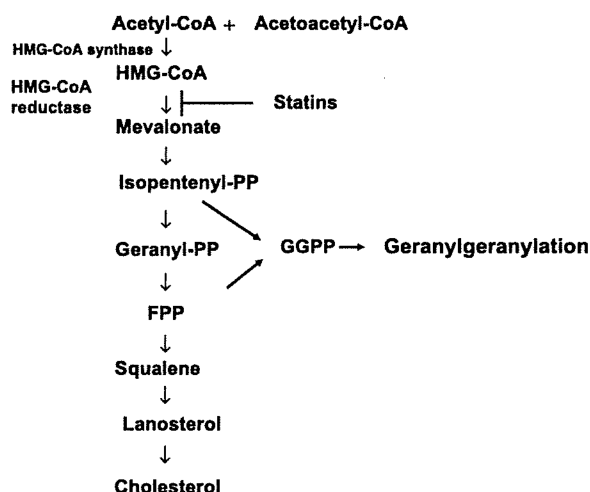

Fig. 2. Cholesterol biosynthesis pathway and statins. In the cholesterol biosynthesis pathway, the production of mevalonate by HMG-CoA reductase is the rate-limiting step. Statins inhibit HMG-CoA reductase, resulting in the inhibition of the production of isoprenoids as well as cholesterol. Geranyl-PP: geranylpyrophosphate and GGPP: geranylgeranylpyrophosphate.

nylgeranyl pyrophosphate (GGPP) are mevalonate-derived isoprenoids (Fig. 2). The attachment of isoprenoid to the cellular proteins is called prenylation. Prenylation regulates a variety of cellular functions, including growth, differentiation, and oncogenesis. From the aspect of the pleiotropic effect of the statins, Wang et al. (19) recently identified FBL2 as geranylgeranylated cellular protein required for HCV RNA replication. FBL2 belongs to the FBL family of proteins, all of which contain an F box and a multiple leucine-rich repeat. These two possible inhibitory mechanisms are proposed for the anti-HCV effect of statins. The low-density lipoprotein receptor (LDLR) is reported as one of the potential HCV receptors (20). However, the precise role of LDLR for HCV is still controversial (21). It will be worth trying to examine the effect of statins in the JFH-1 infection system since statins enhance the expression of LDLR.

Sphingolipid is another major component of lipid rafts and thereby is also the antiviral target for HCV. Serine palmitoyltransferase (SPT) is the enzyme responsible for the condensation of L-serine with palmitoyl-CoA to produce 3-ketodihydrosphingosine in the first step of sphingolipid biosynthesis. Sakamoto et al. (22) and Umehara et al. (23) reported that myriocin, a selective inhibitor of SPT, inhibited the HCV RNA replication in replicon-harboring cells and in HCV-infected chimeric mice with humanized livers, respectively. These results further support the significance of lipid metabolism in HCV RNA replication.

Other than cholesterol and sphingolipid biosynthesis, fatty acids are reported to be metabolites that affect HCV RNA replication. Leu et al. (24) reported that polyunsaturated fatty acids (PUFAs) possessed an anti-HCV effect using HCV-replicon harboring cells. Arachidonic acid, docosahexaenoic acid, linoleic acid, and eicosapentaenoic acid belonging to PUFAs possessed anti-HCV activity. On the other hand, saturated fatty acids enhanced HCV RNA replication. The precise mechanisms of fatty acids regarding HCV RNA replication have remained unclear. Very recently, we examined the effect of ordinary nutrients on HCV RNA replication using the OR6 system (25). Interestingly, we found that vitamin E negated the anti-HCV effect of linoleic acid (25). Given that linoleic acid and vitamin E are an oxidant and antioxidant, respectively, oxidative stress may be involved in HCV RNA replication. Further study in this field will provide clues for developing anti-HCV reagents.

Different anti-HCV effects of statins

Statins are one of the most worldwide used reagents for the treatment of hypercholesterolemia and they are beneficial in the prevention of coronary heart disease. In the cholesterol biosynthesis pathway, the production of mevalonate by HMG-CoA reductase is the rate-limiting step. Statins inhibit mevalonate synthesis by inhibiting HMG-CoA reductase, resulting in decreased production of isoprenoids as well as cholesterol. The activities of some cellular proteins are regulated by the attachment of isoprenoids (prenylation). For example, statins inhibited the function of small G proteins, Ras and Rho. Ras and Rho are major substrates for prenylation with FPP and GGPP, respectively. So far, among the statins, lovastatin is the only one with a well-characterized inhibitory effect against HCV RNA replication in cell culture (17, 26, 27). Recently, FBL2 was identified as one of the of geranylgeranylated cellular proteins required for HCV RNA replication (19). Geranylgeranyrated FBL2 binds to NS5A of HCV and the resulting complex is required for HCV RNA replication (19).

The anti-HCV effect of the statins other than lovastatin remains to be clarified. Therefore, we used the OR6 system to test anti-HCV effect of five statins: lovastatin, simvastatin, atorvastatin, fluvastatin, and pravastatin (2). More recently, we also added pitavastatin to this list, so that finally six statins were tested for their effects on HCV RNA replication. None of the statins exhibited cytotoxicity at the concentrations tested. The 50% effective concentrations (EC_{50}) of statins are summarized in Table 1. The anti-HCV effects of simvastatin, atorvastatin, fluvastatin, and pitavastatin

Table 1. EC_{50} of statins on HCV RNA replication

Statins	EC_{50} (μM)
Lovastatin	2.16
Simvastatin	1.57
Atorvastatin	1.39
Fluvastatin	0.90
Pitavastatin	0.45

were stronger than that previously reported for lovastatin. The EC_{50} of lovastatin, simvastatin, atorvastatin, fluvastatin, and pitavastatin were 2.16, 1.57, 1.39, 0.90, and 0.45 μM, respectively. Pitavastatin possessed the strongest anti-HCV activity among the statins tested and its EC_{90} was calculated as 1.25 μM (Fig. 3A). In contrast, pravastatin exhibited no anti-HCV effect. Pravastatin is the only hydrophilic statin among the statins tested and does not cross the cellular membrane passively. It has been reported that a human liver-specific organic anion transporter, LST-1, mediates the uptake of pravastatin in human hepatocytes (28). Therefore, we examined the expression levels of LST-1 in OR6 cells. OR6 cells expressed the mRNA of LST-1 at levels equivalent to that in normal human liver (2). We ruled out the possibility that pravastatin didn't actually work as the inhibitor for HMG-CoA reductase in the cells. We confirmed that pravastatin induced HMG-CoA reductase by a positive feedback mechanism in response to the decrease of cholesterol by the inhibition of HMG-CoA reductase by pravastatin (2). These results suggest that there may be another mechanism underlying the depletion of GGPP and cholesterol by statins. One of the clues for resolving this puzzle is that pravastatin has a different effect on P450 induction compared with the other statins (29). However, further study will be needed to clarify this issue.

Statins in combination with IFN

The combination therapy of PEG-IFN and ribavirin is a current standard therapy for patients with CH-C. Ribavirin by itself possessed no anti-HCV effect for the patients. However, ribavirin alone exhibited an anti-HCV effect in the OR6 cell culture system when it was used at a concentration higher than that in the serum of patients undergoing ribavirin treatment. The EC_{50} of ribavirin is calculated as 76 μM in the OR6 system and this is approximately 5 – 7 times higher concentration than that in serum from the patients with ribavirin treatment (3). Furthermore, the synergistic effect of ribavirin at the low concentration with IFN was also confirmed in different cell culture systems, including the OR6 system (3, 30, 31). These results suggest that ribavirin works as a kind of the adjuvant for IFN at the low concentration.

To test the effect of statins in combination with IFN-α on HCV RNA replication, we treated the OR6 cells with

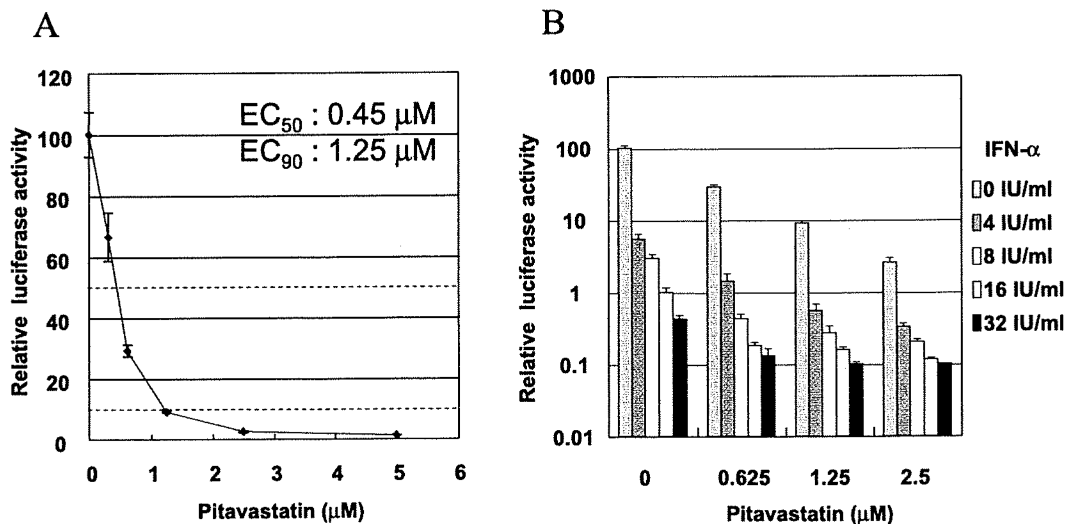

Fig. 3. Anti-HCV effect of pitavastatin in combination with IFN-α. A: OR6 cells were treated with pitavastatin at concentrations of 0, 0.625, 1.25, 2.5, and 5 μM for 72 h. The EC_{50} and EC_{90} were calculated from the result. Shown here is the relative luciferase activity (%) calculated when the luciferase activity of untreated cells was assigned as 100%. B: The effect of pitavastatin in combination with IFN-α. OR6 cells were treated with pitavastatin (0, 0.625, 1.25, and 2.5 μM) and IFN-α (0, 4, 8, 16, and 32 IU/ml) for 72 h. The relative luciferase activity was calculated as shown above.

pitavastatin (0, 0.625, 1.25, and 2.0 μM) and IFN-α (0, 4, 8, 16, and 32 IU/ml) (Fig. 3B). Pitavastatin enhanced the anti-HCV effect of IFN-α in a dose-dependent manner for a fixed concentration of IFN-α, 0, 4, 8, 16, or 32 IU/ml (Fig. 3B). Furthermore, we observed the decrease of luciferase activity to almost the background level in the OR6 reporter assay when OR6 cells were co-treated with 32 IU/ml of IFN-α and pitavastatin at the concentration of 1.25 or 2.5 μM (Fig. 3B). The concentrations of the statins tested in the cell culture were higher than that in the sera from patients with statin administration. However, the statins may enhance the anti-HCV effect of IFN for patients with CH-C at a lower concentration than the EC_{50} in cell culture. Recently O'Leary et al. (32) reported that the monotherapy of atorvastatin does not exhibit anti-HCV activity in a pilot clinical trial. Although the monotherapy of statin seems to be insufficient for patients with CH-C, statin may be a candidate for the adjuvant of IFN therapy like ribavirin.

Conclusions

The OR6 system was developed for the precise and quantitative assay of HCV RNA replication in cell culture. The statins were compared for their anti-HCV effects using the OR6 system and were found to possess different effects on HCV RNA replication. Lovastatin, simvastatin, atorvastatin, fluvastatin, and pitavastatin had different anti-HCV profiles in cell culture. However, pravastatin had no anti-HCV effect, although it worked as inhibitor for HMG-CoA reductase. Pitavastatin exhibited the strongest anti-HCV effect (EC_{50}: 0.45 μM) among the statins tested and enhanced the effect of IFN-α. It may be difficult to achieve the cell culture based EC_{50} of statins in patients with CH-C. However, statins at lower concentration than the EC_{50} in cell culture may enhance the anti-HCV effect of IFN-α in patients with CH-C. Therefore, statins may be suitable as an adjuvant of IFN-α like ribavirin rather than for monotherapy. Lipid metabolism including cholesterol, sphingolipid, and fatty acid biosynthesis seems to be an attractive field for the development of antiviral reagents for HCV.

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ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 2007, p. 2016–2027
0066-4804/07/$08.00+0 doi:10.1128/AAC.01426-06
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Vol. 51, No. 6

Comprehensive Analysis of the Effects of Ordinary Nutrients on Hepatitis C Virus RNA Replication in Cell Culture[▽]

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Received 15 November 2006/Returned for modification 1 February 2007/Accepted 29 March 2007

To date, only a limited number of studies have reported finding an influence of ordinary nutrients on hepatitis C virus (HCV) RNA replication. However, the effects of other nutrients on HCV RNA replication remain largely unknown. We recently developed a reporter assay system for genome-length HCV RNA replication in hepatoma-derived HuH-7 cells (OR6). Here, using this OR6 assay system, we comprehensively examined 46 nutrients from four nutrient groups: vitamins, amino acids, fatty acids, and salts. We found that three nutrients—β-carotene, vitamin D_2, and linoleic acid—inhibited HCV RNA replication and that their combination caused additive and/or synergistic effects on HCV RNA replication. In addition, combined treatment with each of the three nutrients and interferon alpha or beta or fluvastatin inhibited HCV RNA replication in an additive manner, while combined treatment with cyclosporine synergistically inhibited HCV RNA replication. In contrast, we found that vitamin E enhanced HCV RNA replication and negated the effects of the three anti-HCV nutrients and cyclosporine but not those of interferon or fluvastatin. These results will provide useful information for the treatment of chronic hepatitis C patients who also take anti-HCV nutrients as an adjunctive therapy in combination with interferon. In conclusion, among the ordinary nutrients tested, β-carotene, vitamin D_2, and linoleic acid possessed anti-HCV activity in a cell culture system, and these nutrients are therefore considered to be potential candidates for enhancing the effects of interferon therapy.

Hepatitis C virus (HCV) is a major pathogen of chronic hepatitis (CH) and leads to fatal liver diseases, such as liver cirrhosis and hepatocellular carcinoma (1, 23, 38). Approximately 170 million people worldwide are infected with HCV (40). Therefore, HCV infection is a global health problem. The combination of pegylated interferon (IFN) with ribavirin is currently the most effective therapy for CH C (11, 28), and long-term treatment has been shown to improve the sustained virological response (SVR) rate (37). However, the SVR rate still remains at approximately 55% (11). Combination therapy occasionally also causes adverse effects, such as severe anemia (10) and cerebral vascular lesions (7), and reduction of the dosage leads to insufficient treatment. These adverse effects are more serious in older patients. Therefore, it remains necessary to identify alternative agents that have fewer side effects to couple with IFN. In developing countries, it is difficult to administer expensive IFN therapy. Hence, in such countries, inexpensive anti-HCV reagents are especially desirable (36).

The lack of a small-animal model and a cell culture system to support efficient HCV RNA replication hampered the development of anti-HCV reagents. Since an HCV subgenomic replicon system was developed in 1999 (25), the mechanism of HCV RNA replication has been gradually elucidated by a number of groups (2). However, this subgenomic replicon system may not necessarily reflect actual HCV RNA replication in hepatocyte cell lines, due to the absence of structural proteins. To overcome this problem, a cell culture system for genome-length HCV RNA replication was developed by several groups (4, 15, 33). We also developed a genome-length HCV RNA (strain O of genotype 1b) replication system (OR6) with luciferase as a reporter, which facilitated the prompt and precise monitoring of HCV RNA replication in hepatocyte cell lines (13, 30). In the OR6 assay system, the luciferase activity was well correlated with HCV RNA levels when cells were treated with IFN-α (13). Therefore, we quantified the luciferase activity instead of HCV RNA for the indirect evaluation of HCV RNA replication, although the OR6 assay system doesn't strictly quantify the HCV RNA replication. More recently, several groups have developed cell culture systems that produce infectious viral particles (genotype 2a), which can be used to reconstruct the life cycle of HCV infection in hepatocyte cell lines (24, 39, 45).

Using this OR6 assay system, we demonstrated that mizoribine (30), as an immunosuppressant, and fluvastatin (FLV) (14), as the reagent for hypercholesterolemia, inhibited HCV RNA replication in hepatocyte cell lines. Another immunosuppressant, cyclosporine (CsA), was also identified as an anti-HCV reagent in a subgenomic replicon system (41). These results suggested the expansion of the primary application of the existing therapeutic drugs to new anti-HCV therapy.

Other studies have also revealed that certain ordinary nutrients contained in common foods can influence HCV RNA replication (5, 8, 9, 17, 21, 26). However, in these studies, only a limited number of nutrients were tested. To date, there has

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▽ Published ahead of print on 9 April 2007.

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FIG. 1. Schematic gene organization of genome-length HCV RNA. (A) ORN/C-5B/KE RNA replicated in OR6 cells. RL (RLuc) is expressed as a fusion protein with neomycin phosphotransferase (NeoR). The position of an adaptive mutation, K1609E, is indicated by a black triangle. (B) Authentic HCV RNA, HCV-O/KE/EG, was introduced into the OR6c cells by electroporation as previously described (13). The positions of two adaptive mutations, E1202G and K1609E, are indicated by black triangles. IRES, internal ribosome entry site; EMCV, encephalomyocarditis virus.

been no comprehensive analysis of the effects of nutrients on HCV RNA replication. Thus, we inclusively investigated the effects of nutrients on genome-length HCV RNA replication using the OR6 assay system. Here, we report finding that three nutrients, namely, β-carotene (BC), vitamin D_2 (VD2), and linoleic acid (LA), inhibited HCV RNA replication and that combination treatments including each of these nutrients and CsA synergistically inhibited HCV RNA replication. Furthermore, we found that vitamin E (VE) enhanced HCV RNA replication, and that the anti-HCV activities of each of the three nutrients and CsA were abrogated by VE.

MATERIALS AND METHODS

Reagents. Vitamin B_{12}, vitamin K_1 (VK1), vitamin K_3, elaidic acid, and vaccenic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). BC, vitamin A (VA), vitamin B_1, vitamin B_2, vitamin B_3, vitamin B_6, vitamin C (VC), VD2, vitamin D_3 (VD3), VE, vitamin K_2 (VK2), pantothenic acid, biotin, folic acid, inositol, leucine, isoleucine, valine, tryptophan, phenylalanine, tyrosine, lauric acid, palmitic acid, stearic acid, behenic acid, oleic acid, elaidic acid, LA, arachidonic acid (AA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), $Fe(II)SO_4$, Na_2SeO_4, $Fe(III)(NO_3)_3$, $ZnCl_2$, NaCl, KCl, $CaCl_2$, PCl_3, $MgCl_2$, $CuCl_2$, $MnCl_2$, and IFN-α were purchased from Sigma (St. Louis, MO), and CsA and FLV were purchased from Calbiochem (Los Angeles, CA). IFN-β was kindly provided by Toray Industries, Inc. Fatty acids, except for LA-albumin and oleic acid-albumin compounds (Sigma), were compounded with fatty acid-free bovine serum albumin (Sigma) as described in a previous report (6). We treated the HCV RNA-harboring cells at various concentrations of salts [$Fe(II)SO_4$ at 5, 25, and 50 μM, $Fe(III)(NO_3)_3$ at 10, 100, and 200 μM, $ZnCl_2$ at 20, 50, and 100 μM, Na_2SeO_4 at 1, 2.5, and 5 μM, NaCl at 100, 150, and 300 μM, KCl at 5, 10, and 20 μM, $CaCl_2$ at 2, 4, and 8 μM, PCl_3 at 1, 2.5, and 5 μM, $MgCl_2$ at 0.5, 2.5, and 5 μM, $CuCl_2$ at 20, 50, and 100 μM, and $MnCl_2$ at 30, 60, and 120 μM]. Dimethyl sulfoxide or ethanol was used for the dissolution of the liposoluble nutrients, and the final concentrations (0.1%) of each were equal for the cells with nutrients and those without.

Cell cultures. OR6 cells, polyclonal genome-length HCV RNA (ORN/C-5B/KE)-replicating cells, and subgenomic replicon (ORN/3-5B/KE) cells, which were derived from hepatoma cell line, HuH-7, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, streptomycin (designated as the control medium in this study), and G418 (300 μg/ml) (Geneticin; Invitrogen, Carlsbad, CA), and the cells were passaged twice a week at a 5:1 split ratio. ORN/C-5B/KE and ORN/3-5B/KE are derived from HCV-O (strain O of genotype 1b). OR6c cells are cured OR6 cells from which

TABLE 1. Summary of anti-HCV activities of various nutrients

Nutrient type	Nutrient(s) with the indicated characteristic for HCV[a]		
	Inhibitory	Enhancing	Ineffective
Vitamins			
Liposoluble	BC, VD2	VA (retinol), VE, VK1, VK2, VK3	VD3
Water soluble		VC	*VB1*, *VB2*, *VB3* (*niacin*), *VB6*, VB12, *pantothenic acid*, biotin, *folic acid*, *inositol*
Amino acids			
Branched-chain			*Leucine*, *isoleucine*, *valine*
Aromatic		*Tryptophan*	*Phenylalanine*, *tyrosine*
Fatty acids			
Saturated			Lauric acid (C_{12}), palmitic acid (C_{16}), stearic acid (C_{18}), behenic acid (C_{22})
Mono-unsaturated			Oleic acid (C_{18}; 9-unsaturated), elaidic acid (C_{18}; *trans*-form of oleic acid), vaccenic acid (C_{18}; 11-unsaturated)
Polyunsaturated	LA ($C_{18:2}$, n-6), AA ($C_{20:4}$, n-6), EPA ($C_{20:5}$, n-3), DHA ($C_{22:6}$, n-3)		
Salts	$Fe(II)SO_4$, *$Fe(III)(NO_3)_3$*, $ZnCl_2$	Na_2SeO_4	*NaCl*, *KCl*, *$CaCl_2$*, *PCl_3*, *$MgCl_2$*, $CuCl_2$, $MnCl_2$

[a] Nutrients already contained in the medium are indicated in italics. VB1, vitamin B_1; VB2, vitamin B_2; VB3, vitamin B_3; VB6, vitamin B_6; VB12, vitamin B_{12}; VK3, vitamin K_3.

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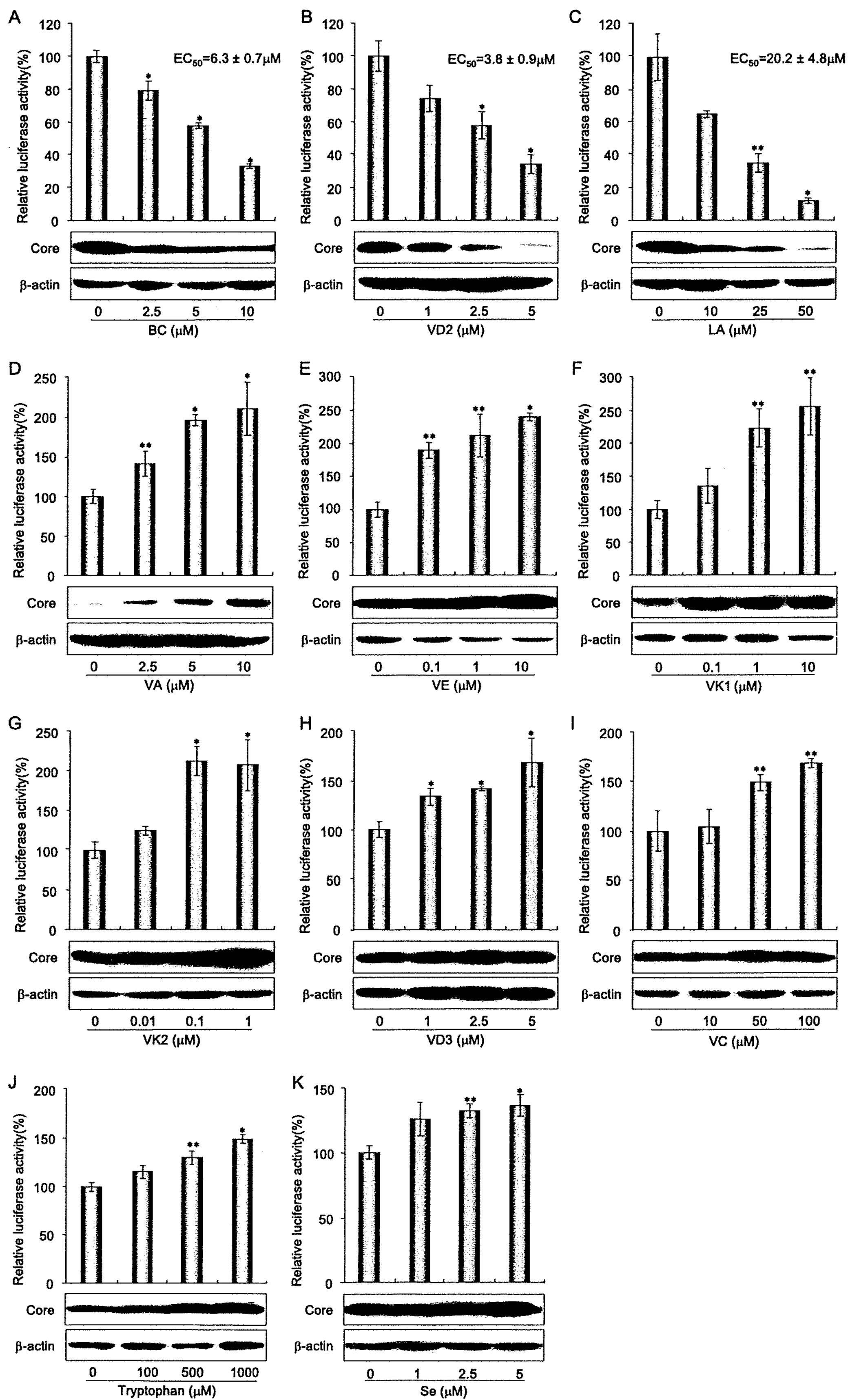

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genome-length HCV RNA was eliminated by IFN-α treatment (500 IU/ml for 2 weeks) without G418, as previously described (13).

Luciferase reporter assay. For the *Renilla* luciferase (RL) assay, approximately 1.0×10^4 to 1.5×10^4 OR6 cells (72-hour treatment) or 0.5×10^4 OR6 cells (120-hour treatment) were plated onto 24-well plates in triplicate and cultured for 24 h. The cells were treated with each nutrient or compound for 72 or 120 h. Then, the cells were harvested with *Renilla* lysis reagent (Promega, Madison, WI) and subjected to the RL assay according to the manufacturer's protocol.

Western blot analysis. For Western blot analysis, 4×10^4 to 4.5×10^4 OR6c cells harboring HCV-O/KE/EG (strain O of genotype 1b) (K. Abe, M. Ikeda, and N. Kato, unpublished data) were plated onto six-well plates and cultured for 24 h and then were treated with each nutrient or compound for 72 h. Preparation of the cell lysates, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotting were then performed as previously described (18). The antibodies used in this study were those specific to HCV core antigen (CP11; Institute of Immunology, Tokyo) and β-actin (Sigma). The epitope of CP11 was located within amino acid positions 21 to 40 of the core antigen. Immunocomplexes on the membranes were detected by enhanced chemiluminescence assay (Renaissance; Perkin Elmer Life Science, Wellesley, MA).

Cell viability. To examine the suppressive effects of nutrients on OR6 cell viability, approximately 4.5×10^4 to 5×10^4 OR6 cells (72-hour viability assay) or approximately 1×10^4 to 1.5×10^4 cells (120-hour viability assay) were plated onto six-well plates in triplicate and were cultured for 24 h. The cells were treated without nutrients or with each nutrient for 72 or 120 h, and then the number of viable cells was counted after trypan blue dye treatment as previously described (30).

Statistical analysis and synergistic statistics. Differences between the anti-HCV activities of the nutrients at each concentration and controls were tested using Student's *t* test. *P* values of less than 0.05 were considered statistically significant. Then, an isobologram method was used to evaluate the effects of a combination of nutrients or compounds on HCV RNA replication (21). OR6 cells were treated with each combination of nutrients or compounds at various concentrations for 72 h. The 50% effective concentration (EC_{50}) against HCV RNA replication in each combination treatment was analyzed by sigmoid regression, and isoboles of EC_{50} were plotted using the resulting data.

RESULTS

Effects of ordinary nutrients on HCV RNA replication. To date, information about the anti-HCV effects of ordinary nutrients has been limited to only a few studies, and in those studies, a plasmid (26), a subgenomic replicon (21), and recombinant HCV proteins (5, 8, 9) were used in the assays. We recently developed OR6 assay system by the selection after introducing genome-length ORN/C-5B/KE RNA (Fig. 1A) into HuH-7 cells. Our OR6 assay system renders it possible to carry out the prompt and precise evaluation of genome-length HCV RNA replication (13, 30). Therefore, we comprehensively analyzed 46 ordinary nutrients to determine their effects on HCV RNA replication using our novel OR6 assay system (Table 1). The effects of the preexistent nutrients in the medium on HCV RNA replication were under a significant level, because the concentrations of the nutrients already contained in the medium were less than a one-thousandth part of the minimum concentration in the treatment.

We first examined 8 liposoluble vitamins and 10 water-soluble vitamins to investigate their effects on HCV RNA replication. Among the liposoluble vitamins, VA (Fig. 2D), VE (Fig. 2E), and VK (Fig. 2F and G) significantly enhanced HCV RNA replication. However, BC and VD2 significantly inhibited HCV RNA replication in a dose-dependent manner (the mean EC_{50}s ± standard deviations [SDs] were 6.3 ± 0.7 μM and 3.8 ± 0.9 μM, respectively) (Fig. 2A and B). In contrast with VD2, VD3 apparently enhanced relative luciferase activity, but this promotive effect was thought to result from cell proliferation, since the amount of β-actin increased in a manner similar to that of HCV core antigen (Fig. 2H). Most of the water-soluble vitamins exerted no effect on HCV RNA replication (data not shown), while only VC moderately enhanced HCV RNA replication (Fig. 2I).

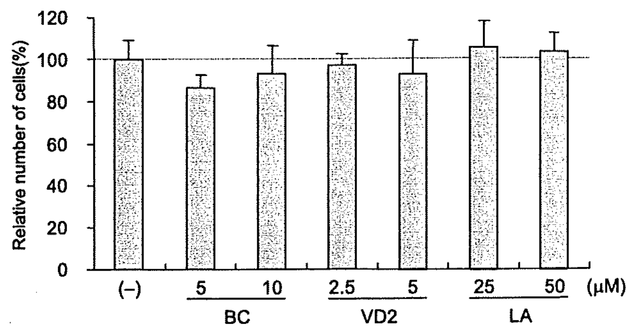

FIG. 3. The anti-HCV activities of three nutrients were not due to the suppression of cell growth. Cell viabilities after treatment with BC, VD2, and LA are shown. OR6 cells were cultured in control medium [(−)] and in the presence of BC (5 and 10 μM), VD2 (2.5 and 5 μM), and LA (25 and 50 μM) for 72 h, and then the number of viable cells was counted after trypan blue dye treatment. Shown here are the percent relative cell numbers calculated when the relative cell number of untreated cells was assigned as 100%. The data indicate means ± SDs of triplicates from at least two independent experiments.

We next examined three branched-chain amino acids and three aromatic amino acids for their effects on HCV RNA replication. We tested the six amino acids at concentrations of 0, 100, 500, and 1,000 μM, and only tryptophan exerted moderate promotive effects on HCV RNA replication (Fig. 2J).

We further examined four saturated fatty acids, three monounsaturated fatty acids, and four polyunsaturated fatty acids (PUFAs) to assess their effects on HCV RNA replication. As has been noted in previous reports (17, 21), all of the PUFAs, i.e., LA, AA, EPA, and DHA, inhibited HCV RNA replication in OR6 cells in a dose-dependent manner (the mean EC_{50}s ± SDs were 20.2 ± 4.8 μM, 22.1 ± 1.7 μM, 36.2 ± 2.5 μM, and 37.0 ± 3.6 μM, respectively). However, we found that with the exception of LA, treatment with 50 μM of PUFA resulted in

FIG. 2. Effects of ordinary nutrients on HCV RNA-replicating cells. (A through K) Reporter assay and Western blot analysis of nutrient sensitivity of HCV RNA replication. OR6 cells were treated with each nutrient at a four-grade-modulated concentration in the medium. After 72 h of treatment, the RL assay was performed as described in Materials and Methods. Shown here are the percent relative luciferase activities calculated when the RL activity of untreated cells was assigned the value of 100%. The data indicate means ± SDs of triplicate samples from at least three independent experiments. Subsequently, OR6c cells, into which authentic HCV RNA was introduced, were treated with nutrients exhibiting either inhibitory effects, i.e., BC (A), VD2 (B), and LA (C), or promotive effects, i.e., VA (D), VE (E), VK1 (F), VK2 (G), VD3 (H), VC (I), tryptophan (J), and Se (K) at the same concentrations as those used in the OR6 assay (bar graphs). After 72 h of treatment, the production of HCV core antigen was analyzed by immunoblotting using antibody specific to HCV core antigen (upper lanes). β-Actin was used as a control for the amount of protein loaded per lane (lower lanes). *, $P < 0.01$; **, $P < 0.05$.

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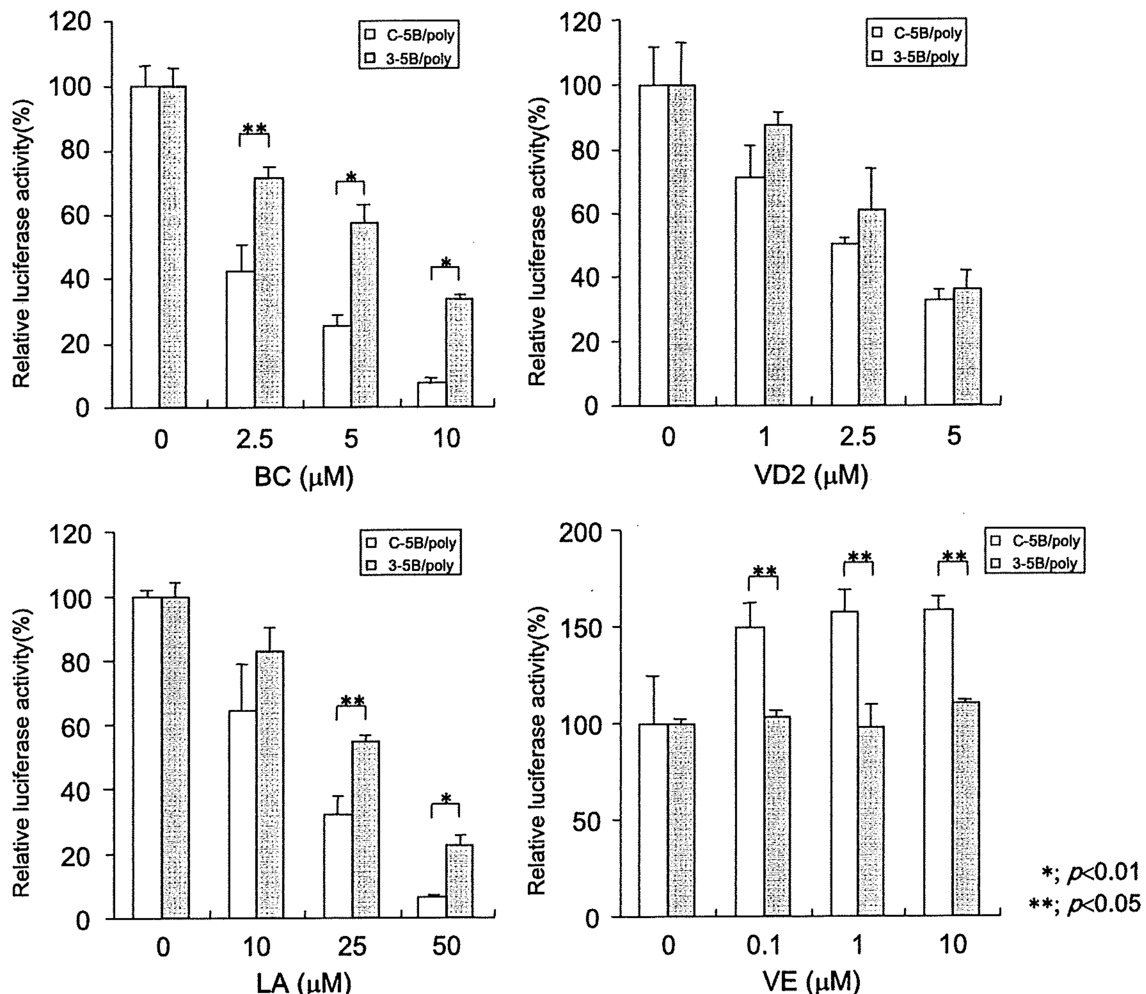

FIG. 4. The effects of BC, VD2, LA, and VE on polyclonal genome-length and subgenomic HCV RNA replication. Both polyclonal genome-length HCV RNA-replicating cells (ORN/C-5B/KE/poly) and subgenomic replicon cells (ORN/3-5B/KE/poly) were treated with BC, VD2, LA, or VE according to the same protocol as that used for the OR6 assay. The RL assay was performed at 72 h postapplication, and then RL activity was calculated as described in the legend to Fig. 2.

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the suppression of cell growth due to cytotoxicity (data not shown). These data indicate that among PUFAs, only LA exhibited a significant inhibitory effect on HCV RNA replication without concomitant cytotoxicity (Fig. 2C and 3).

Finally, we examined 11 salts in order to assess their effects on HCV RNA replication. Iron [Fe(II) in the form of $FeSO_4$ and Fe(III) in the form of $Fe(NO_3)_3$] and zinc (in the form of $ZnCl_2$) exhibited anti-HCV effects without cytotoxicity at concentrations of up to 50% inhibition, but beyond 50% inhibition, cell growth was dose-dependently affected by the cytotoxicity of these minerals. Selenium (in the form of Na_2SeO_4), a typical antioxidant, slightly enhanced HCV RNA replication (Fig. 2K). We also confirmed these results using authentic HCV RNA-replicating cells (Fig. 1B and 2A through K).

These results suggest that the ordinary nutrients tested here have different profiles in terms of their effects on HCV RNA replication. The results are summarized in Table 1. Most of the nutrients were found to have no effect on HCV RNA replication. Eight nutrients enhanced HCV RNA replication, and the antioxidant nutrients VA, VC, VE, and Se were included in this group. Among the 46 nutrients tested with the OR6 assay system, we found that BC, VD2, and LA exerted anti-HCV effects without cytotoxicity. To the best of our knowledge, this is the first study to demonstrate the anti-HCV effects of BC and VD2. Therefore, we focused on the anti-HCV effects of BC, VD2, and LA in the following study.

The effects of BC, VD2, LA, and VE on polyclonal genome-length and subgenomic HCV RNA replication. OR6 cells are among the cloned cell lines selected by G418. Therefore, we examined polyclonal cells harboring genome-length HCV RNA (ORN/C-5B/KE/poly) to exclude the possibility that the anti-HCV effects of BC, VD2, and LA were an OR6 clone-specific phenomenon. Furthermore, polyclonal cells harboring subgenomic HCV RNA (ORN/3-5B/KE/poly) were also used to examine the effects of the anti-HCV nutrients on RNA replication in the absence of the structural HCV proteins. The

results revealed that all of these three nutrients exhibited a dose-dependent suppression of HCV RNA replication in both cell systems, although the three nutrients had stronger anti-HCV effects in the polyclonal genome-length HCV RNA-replicating cells than they did in the subgenomic HCV RNA-replicating cells (Fig. 4). These results indicated that the anti-HCV activities of these nutrients were not due to cell clonality, and the sensitivities of the reagents were found to differ between subgenomic and genome-length HCV RNA-replicating cells. One possible explanation of this difference is that the different genome sizes of subgenomic (9-kb) and genome-length (12-kb) HCV RNA might affect the replication efficiencies and lead to the difference in the sensitivities of antiviral reagents. These differences were significant, especially in BC- and LA-treated cells. A subgenomic replicon system may underestimate the effects of anti-HCV reagents and therefore might fail to identify potentially effective anti-HCV reagents. Therefore, our genome-length HCV RNA replication system (OR6) is advantageous for evaluating anti-HCV candidates.

We also tested VE's effect on subgenomic and genome-length HCV RNA-replicating cells. VE enhanced the replication of genome-length HCV RNA. However, interestingly, VE did not affect subgenomic HCV RNA replication. These results suggest that the subgenomic HCV RNA replication system may not be able to evaluate the reagent-enhancing HCV RNA replication.

Anti-HCV activities of three nutrients were not due to inhibition of cell growth. Since it has been reported that HCV RNA replication is dependent on cell growth (34), we examined whether the anti-HCV activities of three nutrients were due to their respective cytotoxicities. OR6 cells were treated with each nutrient (BC, 5 and 10 μM; VD2, 2.5 and 5 μM; LA, 25 and 50 μM) for 72 h. These results suggest that the anti-HCV effects of BC, VD2, and LA are not due to their cytotoxicities.

Time course assay of inhibitory effects of three nutrients on HCV RNA replication. A kinetics analysis of the anti-HCV effects of reagents provides information about inhibitory mechanisms and optimized drug administration. Therefore, we conducted a time course assay (24, 72, and 120 h after treatment) of the anti-HCV effects of three nutrients, BC, VD2, and LA, using our OR6 assay system. The results revealed that BC and VD2 exhibited stronger inhibition of HCV RNA replication than did LA at 24 h after treatment. However, the anti-HCV activities of BC and VD2 only slightly increased at 72 or 120 h after treatment (Fig. 5A). On the other hand, LA inhibited HCV RNA replication in dose- and time-dependent manners. It is noteworthy that about 90% inhibition of RL activity was observed at 120 h after LA (50 μM) treatment of OR6 cells (Fig. 5A).

We examined whether these reductions in relative RL activity induced by all three nutrients at 120 h were due to the suppression of cell growth. Compared to the number of untreated cells, at 120 h after treatment with each nutrient (BC, 5 and 10 μM; VD2, 2.5 and 5 μM; LA, 25 and 50 μM), no significant reduction in the number of treated cells was observed (Fig. 5B). These results indicate that the anti-HCV effects of these three nutrients were not due to their respective cytotoxicities.

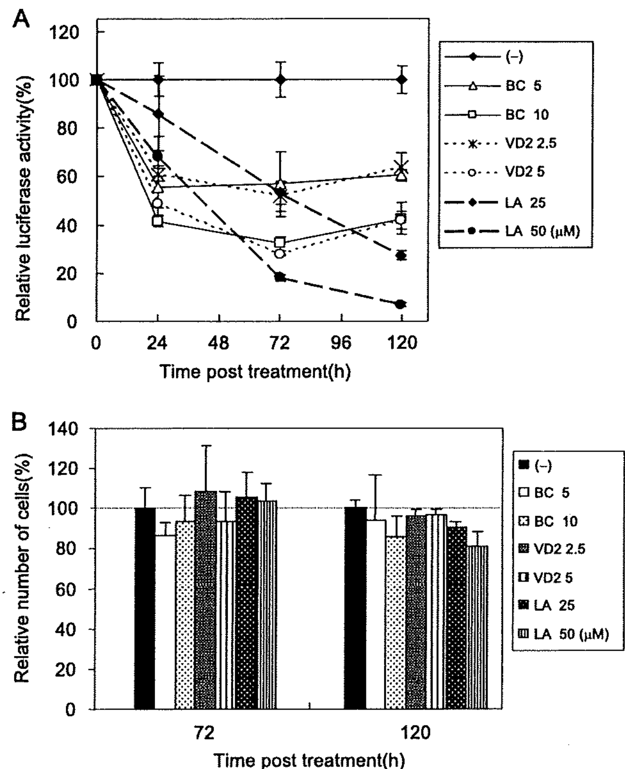

FIG. 5. Time course assay of the anti-HCV activities of three nutrients. (A) Time course of the inhibitory effects of three nutrients on HCV RNA replication. OR6 cells were treated with control medium [(−)], BC (5 and 10 μM), VD2 (2.5 and 5 μM), or LA (25 and 50 μM), and the RL assay was performed at 24, 72, and 120 h postapplication. Relative RL activity was calculated as described in the legend to Fig. 2. (B) Time course of cell viability after the application of three nutrients. OR6 cells were cultured in the control medium and in the presence of BC (5 and 10 μM), VD2 (2.5 and 5 μM), or LA (25 and 50 μM), and at 72 and 120 h postapplication, the number of viable cells was counted after trypan blue dye treatment. Shown here are the percent relative cell numbers calculated as described in the legend to Fig. 3.

HCV RNA replication was additively inhibited by each combination of three nutrients and was synergistically inhibited by all three. As described above, we found that BC, VD2, and LA possessed anti-HCV activities. However, these nutrients appeared to be insufficient for eliminating HCV by mono-treatment. Therefore, we examined the anti-HCV effects of two or three nutrients in combination.

To evaluate the effects of each combination treatment, OR6 cells were cotreated with two nutrients at the listed concentrations for 72 h (BC, approximately 0 to 5 μM; VD2, approximately 0 to 3 μM; LA, approximately 0 to 20 μM). Isoboles of 50% inhibition of HCV RNA replication were obtained for each data point. An analysis of the 50% isoboles of each combination treatment graphed nearly a straight line in each case. These results indicate that the inhibitory effects of all combinations on HCV RNA replication were additive (Fig. 6A).

Treatment with all three nutrients at various concentrations resulted in stronger suppression of HCV RNA replication in OR6 cells than we had predicted as an additive effect (Fig. 6B).

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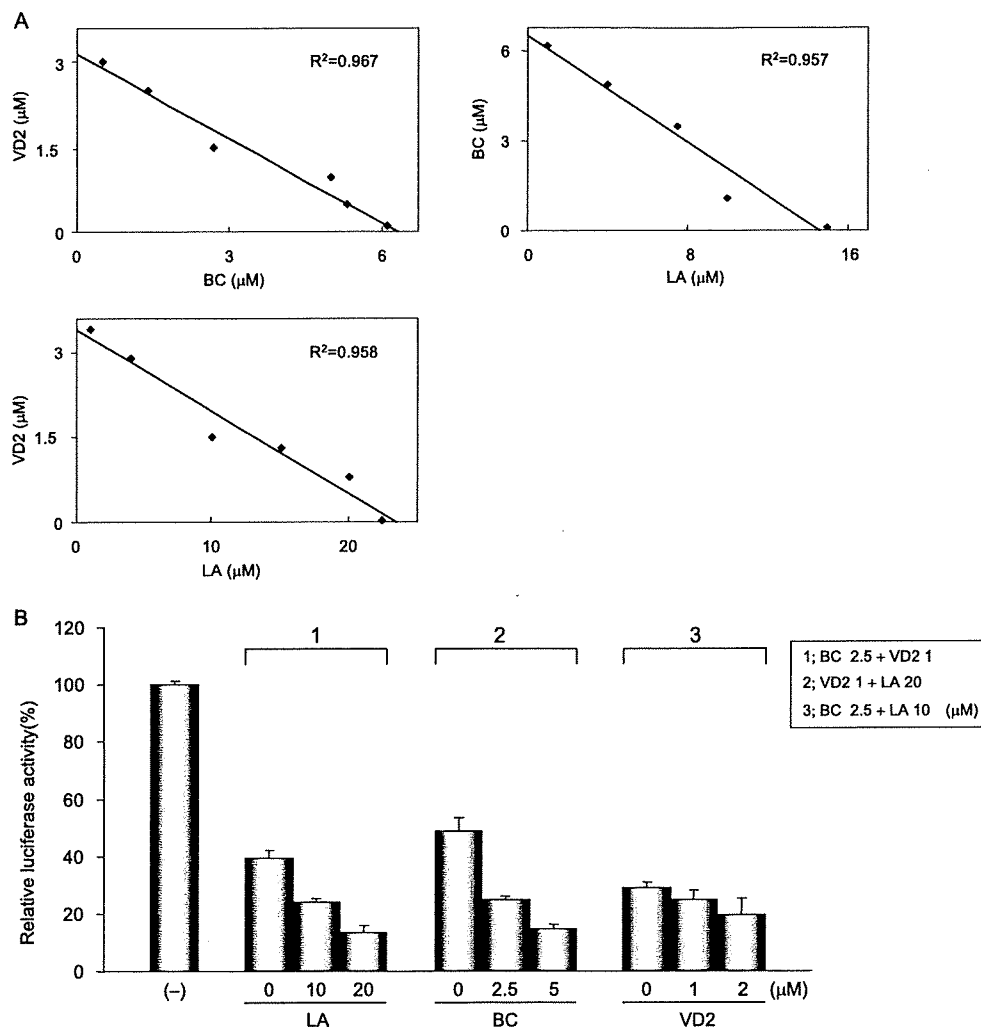

FIG. 6. Effects of treatment with each combination or all of the three nutrients on HCV RNA replication. (A) Isobole plots of 50% inhibition of HCV RNA replication. OR6 cells were treated with each combination of three nutrients, BC (0, 0.5, 1, 2, 3, 4, and 5 μM), VD2 (0, 0.1, 0.5, 1, 1.5, 2, and 3 μM), and LA (0, 1, 5, 10, 15, and 20 μM), for 72 h, and RL assay was performed as described in the legend to Fig. 2 to obtain 50% isoboles. The R^2 value indicates the coefficient of determination. (B) The effect of treatment with all three nutrients on HCV RNA replication was synergistic. OR6 cells were treated with LA (0, 10, and 20 μM) in addition to 2.5 μM BC plus 1 μM VD2, BC (0, 2.5, and 5 μM) in addition to 1 μM VD2 plus 20 μM LA, or VD2 (0, 1, and 2 μM) in addition to 2.5 μM BC plus 10 μM LA. After 72 h of treatment, the RL assay was performed, and then relative RL activity was calculated as described in the legend to Fig. 2.

For instance, in the sample cotreated with 2.5 μM BC ($\approx EC_{20}$) in addition to 1 μM VD2 ($\approx EC_{30}$) plus 20 μM LA ($\approx EC_{50}$) (Fig. 2A through C), the actual effect on HCV RNA replication was 90% inhibition, which was 20% greater than we had originally estimated (i.e., approximately 70%; $1 - 0.8 \times 0.7 \times 0.5 = 0.72$) (Fig. 6B). In addition, no suppression of cell growth was observed during these cotreatments (data not shown). These results suggest that treatment with a mixture of these three nutrients may exert synergistic inhibitory effects on HCV RNA replication.

Treatment with each of three nutrients in combination with IFN or FLV additively inhibited HCV RNA replication, and CsA synergistically inhibited HCV RNA replication. Recently, CsA was proposed as a novel candidate to be paired with IFN in similar studies using a cell culture system (41). We have also reported findings obtained with 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, statins, exerted diverse anti-HCV effects, and FLV was found to exert the strongest inhibitory effect on HCV RNA among the statins tested (14).

Therefore, we examined the anti-HCV effects of each of three nutrients in combination with IFN, FLV, or CsA by using OR6 cells. OR6 cells were treated for 72 h with IFN-α (0, 0.2, 0.5, and 1 IU/ml) in combination with each of the nutrients at various concentrations (BC, approximately 0 to 5 μM; VD2, approximately 0 to 4 μM; LA, approximately 0 to 20 μM) (Fig. 7A). FLV (approximately 0 to 2 μM) or CsA (approximately 0 to 1 μg/ml) was also used for treatment in combination with BC, VD2, or LA at the concentration mentioned above (Fig. 7B and C). Isoboles of 50% inhibition of HCV RNA replication were generated from each sample. An analysis of 50%

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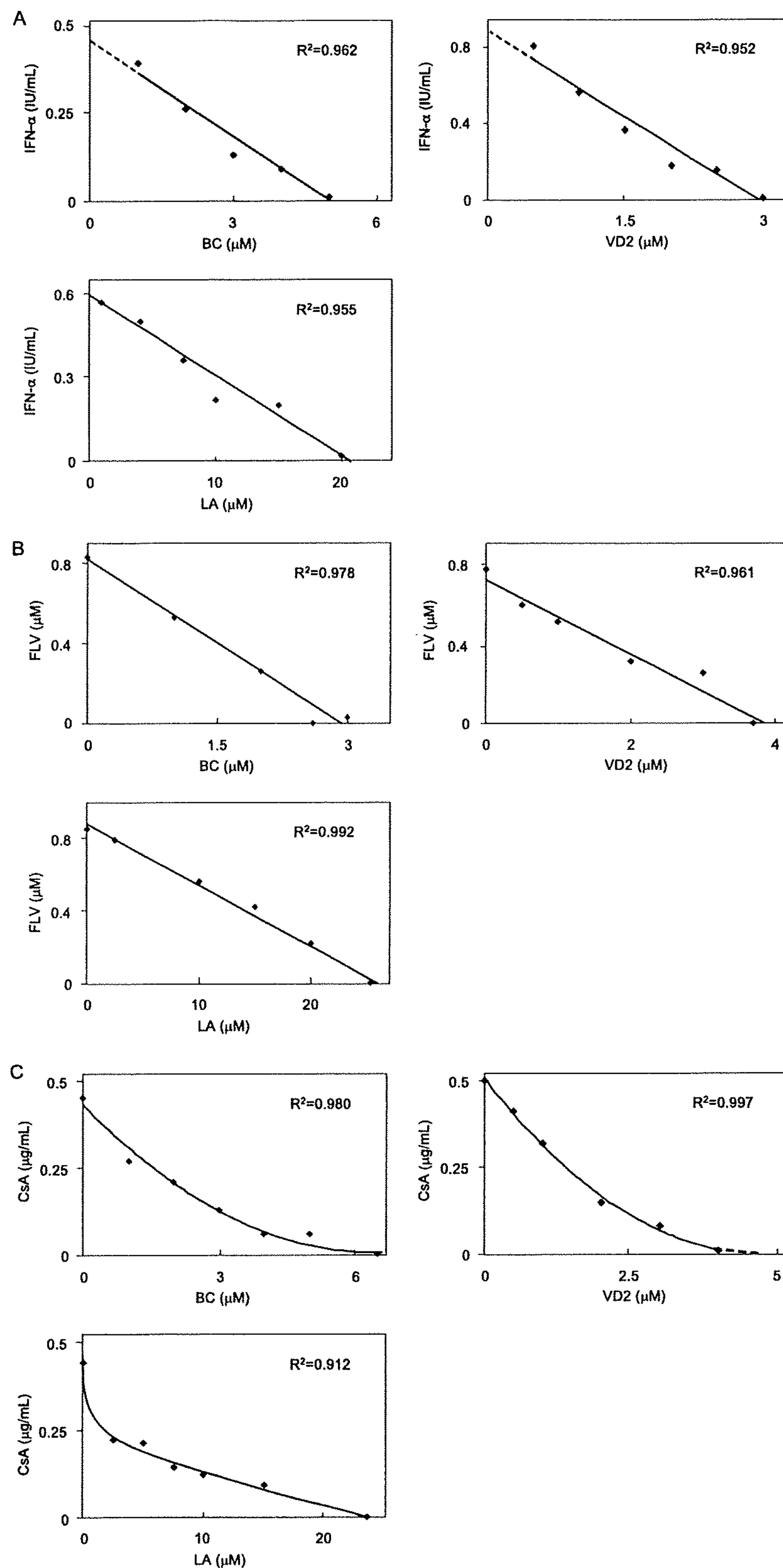

FIG. 7. Additive inhibitory effects of each of three nutrients in combination with IFN-α or FLV on HCV RNA replication, and synergistic effects observed with Cs. (A to C) Isobole plots of 50% inhibition of HCV RNA replication. OR6 cells were treated with BC (0, 1, 2, 3, 4, and 5 μM), VD2 (0, 0.5, 1, 2, 3, and 4 μM), and LA (0, 2.5, 5, 10, 15, and 20 μM) in combination with IFN-α (0, 0.2, 0.5, and 1 IU/ml) (A), FLV (0, 0.5, 1, and 2 μM) (B), or CsA (0, 0.2, 0.5, and 1 μg/ml) (C) for 72 h, and the RL assay was performed as described in the legend to Fig. 2 to obtain 50% isoboles. The R^2 value indicates the coefficient of determination.

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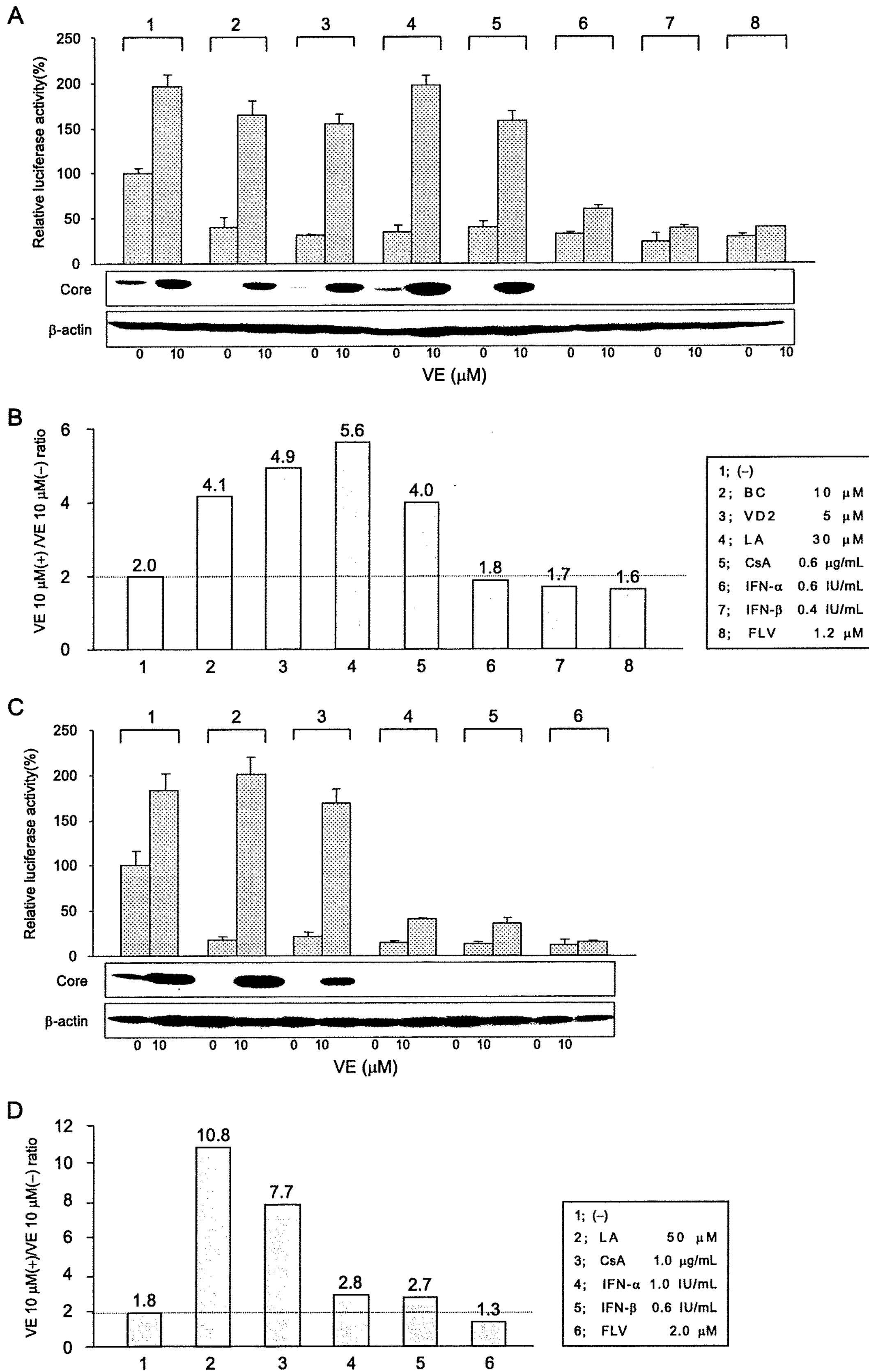

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isoboles in combinations using each nutrient and IFN-α or FLV graphed nearly straight lines in each case, indicating that the suppressive effects of these cotreatments on HCV RNA replication were additive (Fig. 7A and B). Similar additive effects were obtained in combination with IFN-β (data not shown). It was noteworthy that all cotreatments with each nutrient and CsA resulted in curved, concave plots of 50% isoboles, thus suggesting that these combinations with CsA exerted synergistic inhibitory effects on HCV RNA replication (Fig. 7C). These results indicate that these three nutrients, administered as a supportive nutritional therapy, could potentially improve the SVR rate associated with IFN therapy alone.

The anti-HCV activities of BC, VD2, and LA, as well as that of CsA but not those of IFN and FLV, were completely canceled by VE. Among the 46 nutrients tested, BC and VD2 exhibited inhibitory effects on HCV RNA replication up to 70%, and LA exhibited inhibitory effects up to 90%, without exhibiting any cytotoxicity (Fig. 5A). In contrast, most of the liposoluble vitamins enhanced HCV RNA replication in OR6 cells. We used VE in the following studies because VE is one of the most common vitamins in the daily diet and it exerts a strong enhancing effect on HCV RNA replication. To clarify the mechanism of these opposing effects, we investigated whether the anti-HCV effects of BC, VD2, and LA were canceled by the addition of VE. We also tested representative anti-HCV compounds (i.e., CsA, IFN-α, IFN-β, and FLV) in combination with VE. We first examined the influence of 10 μM VE on the nutrients and compounds at the 70% inhibitory concentration level (Fig. 8A and B). The inhibitory effects of IFN-α, IFN-β, and FLV were hardly influenced by cotreatment with VE, whereas the anti-HCV effects of BC, VD2, LA, and CsA were canceled to a significant level by VE in the OR6 cells (Fig. 8A, upper panel). These results were also confirmed using authentic HCV RNA-replicating cells (Fig. 8A, lower panel). To normalize these results, we divided the luciferase value observed in the presence of VE by that in the absence of VE, and we considered this value to represent the effects of VE. When this value was larger than the value obtained in the absence of anti-HCV reagent (2.0; column 1 in Fig. 8B), we interpreted it as indicative of a reagent whose anti-HCV effects were canceled by VE. According to this criterion, BC (4.1), VD2 (4.9), LA (5.6), and CsA (4.0) were evaluated to be reagents for which the anti-HCV effects were canceled by VE (columns 2, 3, 4, and 5 in Fig. 8B). The anti-HCV effects of IFN-α, IFN-β, and FLV were not affected by VE (columns 6, 7, and 8 in Fig. 8B). We next examined the influence of 10 μM VE on the anti-HCV nutrients and compounds at the 90% inhibitory concentration level (Fig. 8C and D). BC and VD2 were not assessed in this experiment, because the maximum inhibitory effect was 70% in the case of these nutrients (Fig. 5A). Similar results were obtained in this experiment. LA (10.8) and CsA (7.7) were evaluated to be reagents for which the anti-HCV effects were canceled by VE (compare columns 2 and 3 to column 1 in Fig. 8D), although IFN-α (2.8) and IFN-β (2.7) were slightly affected by VE at this concentration (Fig. 8D, compare columns 4 and 5 to column 1). Judging by these results, it appears that BC, VD2, LA, and CsA may share some mechanism by which VE negated their anti-HCV activities.

DISCUSSION

The differential effects of BC and VA, as well as those of VD2 and VD3, which belong to the same categories as VA and VD, respectively, are of interest. We observed that whereas BC and VD2 inhibited HCV RNA replication, VA enhanced it, and VD3 exhibited basically no effect. The mechanism governing how these vitamins from the same category exert different effects on HCV RNA remains to be elucidated. However, liposoluble vitamins have been reported to exhibit various physiological activities with each nuclear receptor, consequently acting as hormone-like substances (19, 20, 27, 35). Differences in the gene products induced by each of these vitamins may lead to differences in the effects on HCV RNA replication. Another explanation might be considered in the light of findings suggesting that VA is an antioxidant, and yet recently, BC has been reported to induce oxidative stress (32, 43). This diversity of activities among vitamins in the same category, VA, might result in a variety of influences on HCV RNA replication. Further studies are still needed to account for why these different consequences are generated.

Previous studies have demonstrated that PUFAs such as AA, EPA, and DHA inhibit HCV RNA replication in cell culture systems (17, 21). However, saturated and mono-unsaturated fatty acids have been shown to enhance HCV RNA replication (17). In the prior studies, the cells tolerated the presence of PUFAs at concentrations of up to 50 μM. In contrast, in our study, 50 μM PUFAs were toxic, with the exception of LA. Furthermore, saturated and mono-unsaturated fatty acids hardly exhibited any effects on HCV RNA replication in our OR6 cell culture system. These discrepancies might be due to differences in both the clonalities of the cells and the HCV strains used in each experiment.

FIG. 8. VE canceled the anti-HCV activities of BC, VD2, LA, and CsA. (A and B) Effects of VE on the nutrients and compounds at the 70% inhibitory concentration. Both OR6 cells and OR6c cells, into which authentic HCV RNA was introduced, were treated with control medium [(−)], 10 μM BC, 5 μM VD2, 30 μM LA, 0.6 μg/ml of CsA, 0.6 IU/ml of IFN-α, 0.4 IU/ml of IFN-β, or 1.2 μM FLV in either the absence or presence of 10 μM VE for 72 h. After treatment, an RL assay of harvested OR6 cell samples was performed, and then the relative RL activity was calculated as described in the legend to Fig. 2. Subsequently, the production of HCV core antigen in OR6c cells was analyzed by immunoblotting using antibody specific to HCV core antigen. β-Actin was used as a control for the amount of protein loaded per lane (A). Then, the ratio of RL activity in the presence of 10 μM VE (+) to the RL activity in the absence of VE (−) was calculated. The horizontal line indicates the promotive effect of 10 μM VE alone on HCV RNA replication as a baseline (B). (C and D) Effects of VE on the nutrients and compounds at the 90% inhibitory concentration. Both OR6 cells and OR6c cells were treated with control medium, 50 μM LA, 1 μg/ml of CsA, 1 IU/ml of IFN-α, 0.6 IU/ml of IFN-β, and 2 μM FLV in either the absence (−) or presence (+) of 10 μM VE for 72 h. After treatment, the RL assay and Western blot analysis were performed (C), and then the ratio of RL activity in the presence of 10 μM VE to the RL activity in the absence of VE was calculated in the same manner as that described above (D).

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Here, we demonstrated that three nutrients, BC, VD2, and LA, exhibited anti-HCV effects in polyclonal genome-length and subgenomic HCV RNA (strain O of genotype 1b)-replicating cells. These results indicated that the inhibitory activities of at least three anti-HCV nutrients are not limited to a specific cell clone (OR6).

Moreover, IFN or FLV exhibited additive anti-HCV effects when the cells were cotreated with each of the three anti-HCV nutrients. However, CsA showed synergistic anti-HCV effects in combination with each of these three nutrients. Interestingly, these results coincided with the experiment using VE, as VE canceled the anti-HCV effects of CsA but not those of IFN or FLV. It was recently demonstrated that the anti-HCV effects of CsA are related to the inhibition of cyclophilin (31, 42). CsA is also known as an oxidant that can cause renal or vascular dysfunction, and interestingly, antioxidants, including VE, attenuate these CsA-induced side effects (16, 22). Furthermore, we confirmed that another antioxidant, Se, also weakened the anti-HCV effects of BC, VD2, and LA (data not shown). Therefore, BC, VD2, and LA may possess an anti-HCV mechanism similar to that of CsA, and oxidative stress may be involved in these anti-HCV effects to some extent. Among the nutrients tested, VA, VC, VE, and Se enhanced HCV RNA replication, and these nutrients functioned as antioxidants. In contrast, four PUFAs inhibited HCV RNA replication, and they served as oxidants (29, 44). These results are further evidence of the involvement of oxidative stress in HCV RNA replication.

CH C patients may take excessive doses of VE during the course of interferon therapy, because as an antioxidant, VE has been expected to prevent injury to hepatocytes caused by oxidative stress. However, our results suggest that the potentially negative effects of VE on therapy for CH C patients should be carefully considered. To date, the significance of the role played by ordinary nutrients in viral infections has not been well characterized and has even been underestimated. We believe that our findings will shed light on the field of viral infection from the perspective of the nutritional sciences.

It is difficult to determine the blood concentrations of the nutrients tested in this study because the administration conditions may affect the concentrations in the blood. Rühl et al. (35) reported that the concentrations of BC in human serum are between 0.34 to 0.54 μM and that the average concentration in the human liver is 4.4 μM. Hagenlocher et al. (12) reported that the concentration of LA in human serum is 0.8 to 11.9 μg/100 μl. Armas et al. (3) reported that the concentration of VD2 in human serum at 24 h after a 50,000-IU administration is about 50 nM. The concentration of the nutrient in this study is higher than that in those reports. Therefore, monotreatment of the nutrient may not eliminate HCV. However, these nutrients may boost the effect of IFN treatment in combination like ribavirin does.

It is worth trying to examine the effects of BC, VD2, and LA on the recently developed JFH1 infectious virus production system in a future study. Here, it remains unclear whether these three nutrients affect the production of the virus. Furthermore, the comparison of the effects of these three nutrients between HCV genotypes 1 and 2 will provide useful information for the HCV therapy, as HCV genotypes 1 and 2 respond differentially to IFN treatment.

The precise mechanism underlying the anti-HCV activities of the nutrients has not been clarified in this study. The nutrients may inhibit viral RNAs and proteins, including the internal ribosome entry site, NS3-4A serine protease, and NS5B polymerase. Further in vitro study will be needed to clarify the targets of the nutrients responsible for their anti-HCV activities. Another possibility is that the nutrients inhibit the cellular proteins required for HCV RNA replication. We are now planning a study to clarify the mechanism underlying the nutrients' anti-HCV activities.

In conclusion, we found that three nutrients, BC, VD2, and LA, inhibited HCV RNA replication in a cell culture system and that Se, tryptophan, and various vitamins (A, C, E, and K) enhanced HCV RNA replication. The anti-HCV effects of BC, VD2, and LA were reversed by VE. These results are expected to provide useful information for the improvement of the SVR rates of patients receiving the currently standard IFN therapy. In addition, these findings may contribute to the development of a nutritional supplement specific to the treatment of people with CH C.

ACKNOWLEDGMENTS

We thank Atsumi Morishita for her technical assistance and Yasuo Ariumi for his helpful discussions.

This work was supported by a Grant-in-Aid for the Third-Term Comprehensive 10-Year Strategy for Cancer Control and by a Grant-in-Aid for research on hepatitis, both from the Ministry of Health, Labor, and Welfare of Japan. K.A. was supported by a Research Fellowship from the Japan Society for the Promotion of Science for Young Scientists.

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