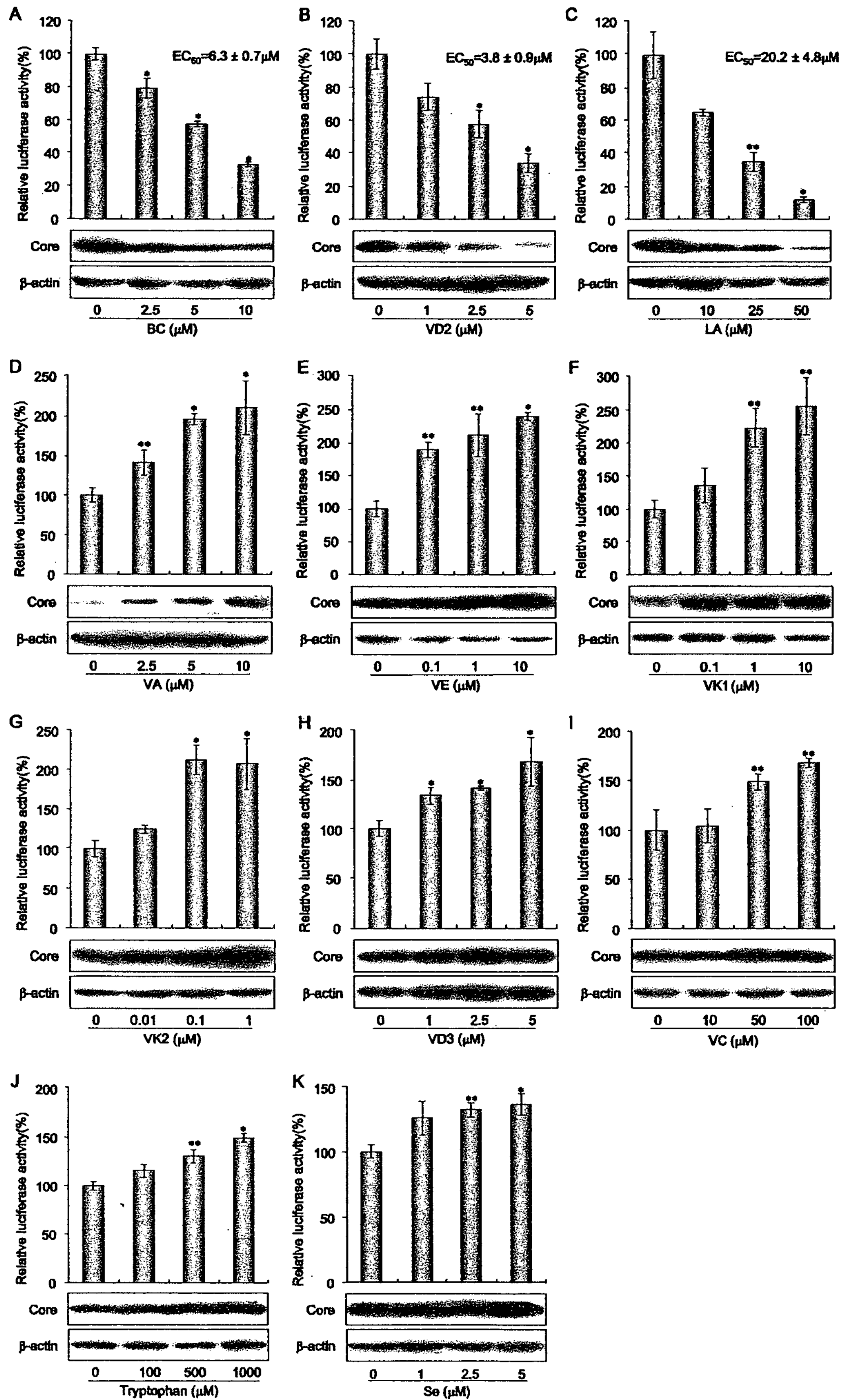

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genome-length HCV RNA was eliminated by IFN-α treatment (500 IU/ml for 2 weeks) without G418, as previously described (13).

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analysis, 4×10^4 to 4.5×10^4 OR6c cells harboring HCV-O/KE/EG (strain O of genotype 1b) (K. Abe, M. Ikeda, and N. Kato, unpublished data) were plated onto six-well plates and cultured for 24 h and then were treated with each nutrient or compound for 72 h. Preparation of the cell lysates,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).

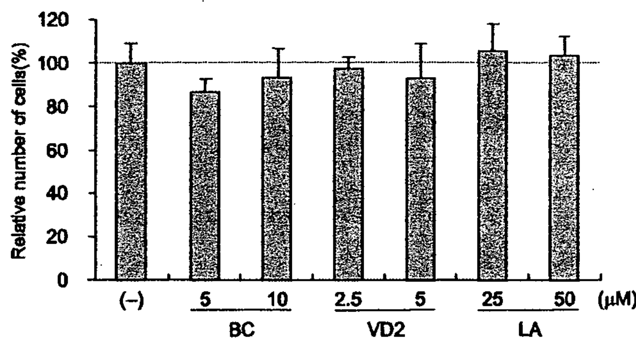

FIG. 3. The anti-HCV activities of three nutrients were not due to the suppression of cell growth. Cell viabilities after treatment with BC, VD2, and LA are shown. OR6 cells were cultured in control medium [(−)] and in the presence of BC (5 and 10 μM), VD2 (2.5 and 5 μM), and LA (25 and 50 μM) for 72 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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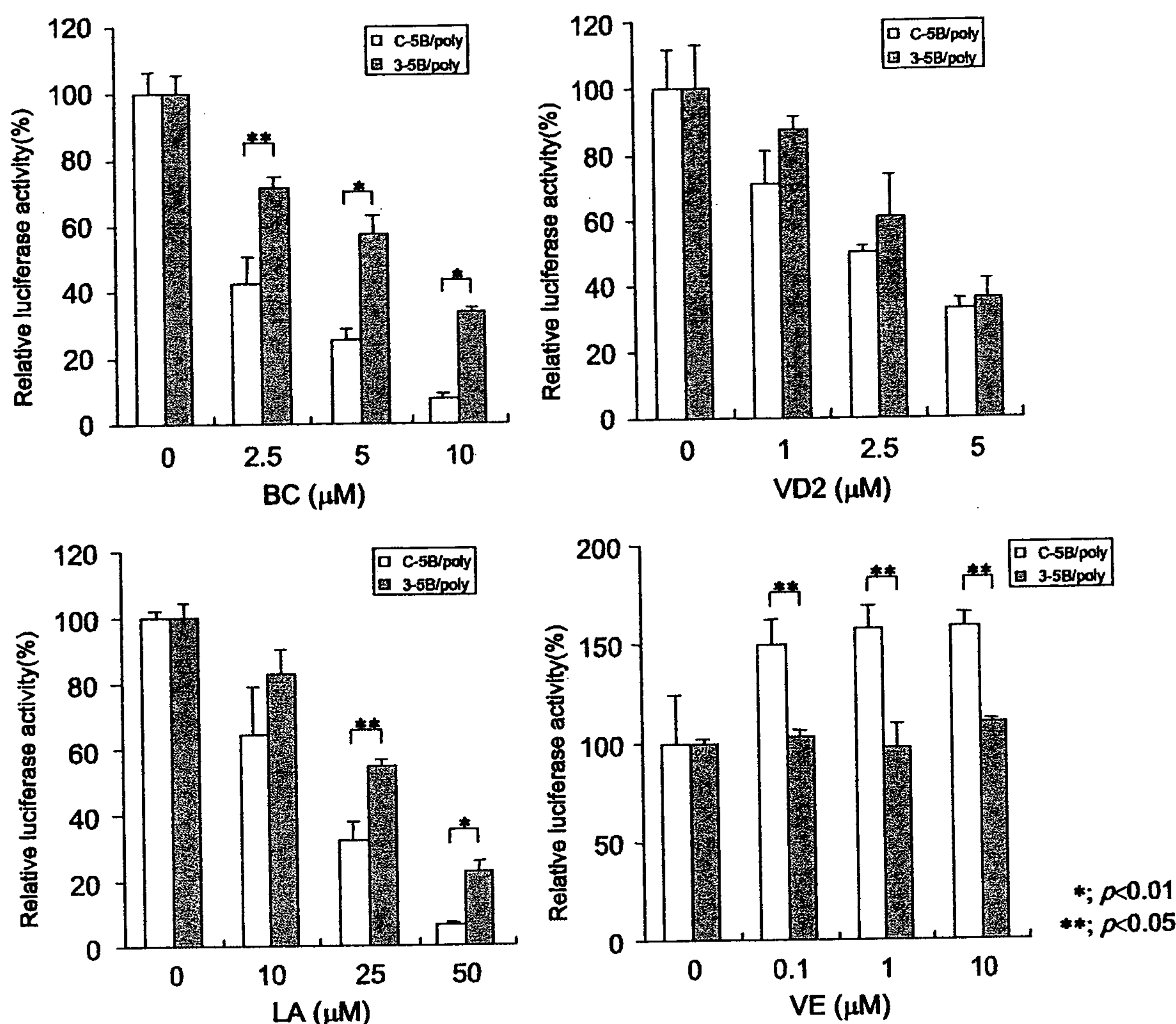

FIG. 4. The effects of BC, VD2, LA, and VE on polyclonal genome-length and subgenomic HCV RNA replication. Both polyclonal genome-length HCV RNA-replicating cells (ORN/C-5B/KE/poly) and subgenomic replicon cells (ORN/3-5B/KE/poly) were treated with BC, VD2, LA, or VE according to the same protocol as 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.

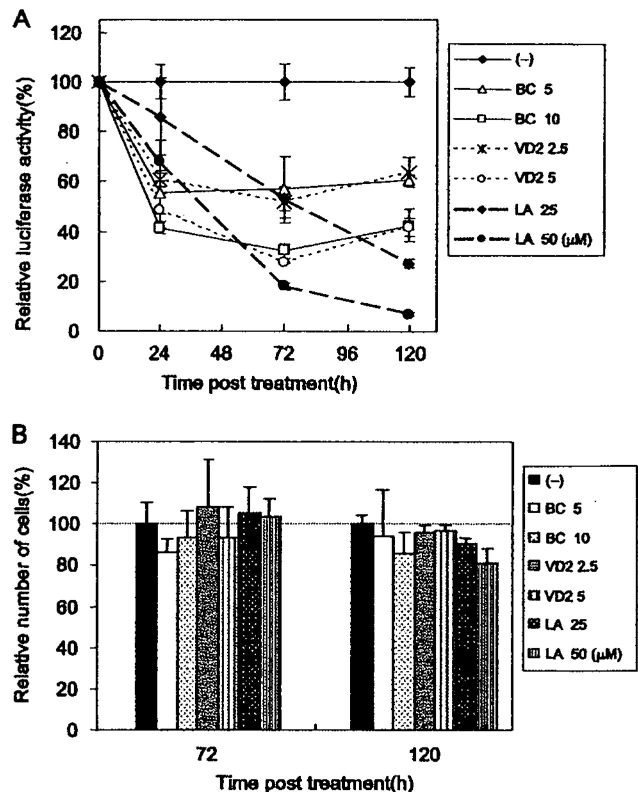

FIG. 5. Time course assay of the anti-HCV activities of three nutrients. (A) Time course of the inhibitory effects of three nutrients on HCV RNA replication. OR6 cells were treated with control medium [(−)], BC (5 and 10 μM), VD2 (2.5 and 5 μM), or LA (25 and 50 μM), and the RL assay was performed at 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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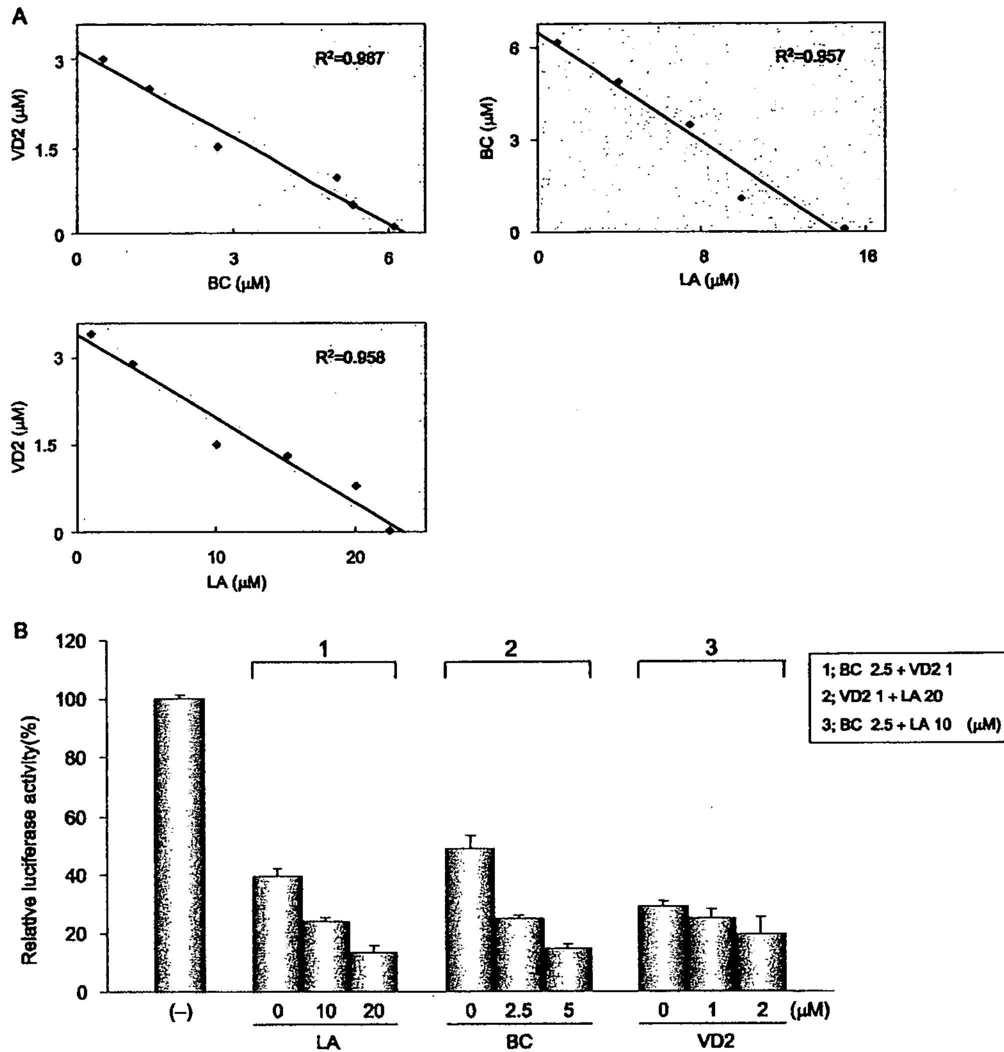

FIG. 6. Effects of treatment with each combination or all of the three nutrients on HCV RNA replication. (A) Isobole plots of 50% inhibition of HCV RNA replication. OR6 cells were treated with each combination of three nutrients, BC (0, 0.5, 1, 2, 3, 4, and 5 μM), VD2 (0, 0.1, 0.5, 1, 1.5, 2, and 3 μM), 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.

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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%

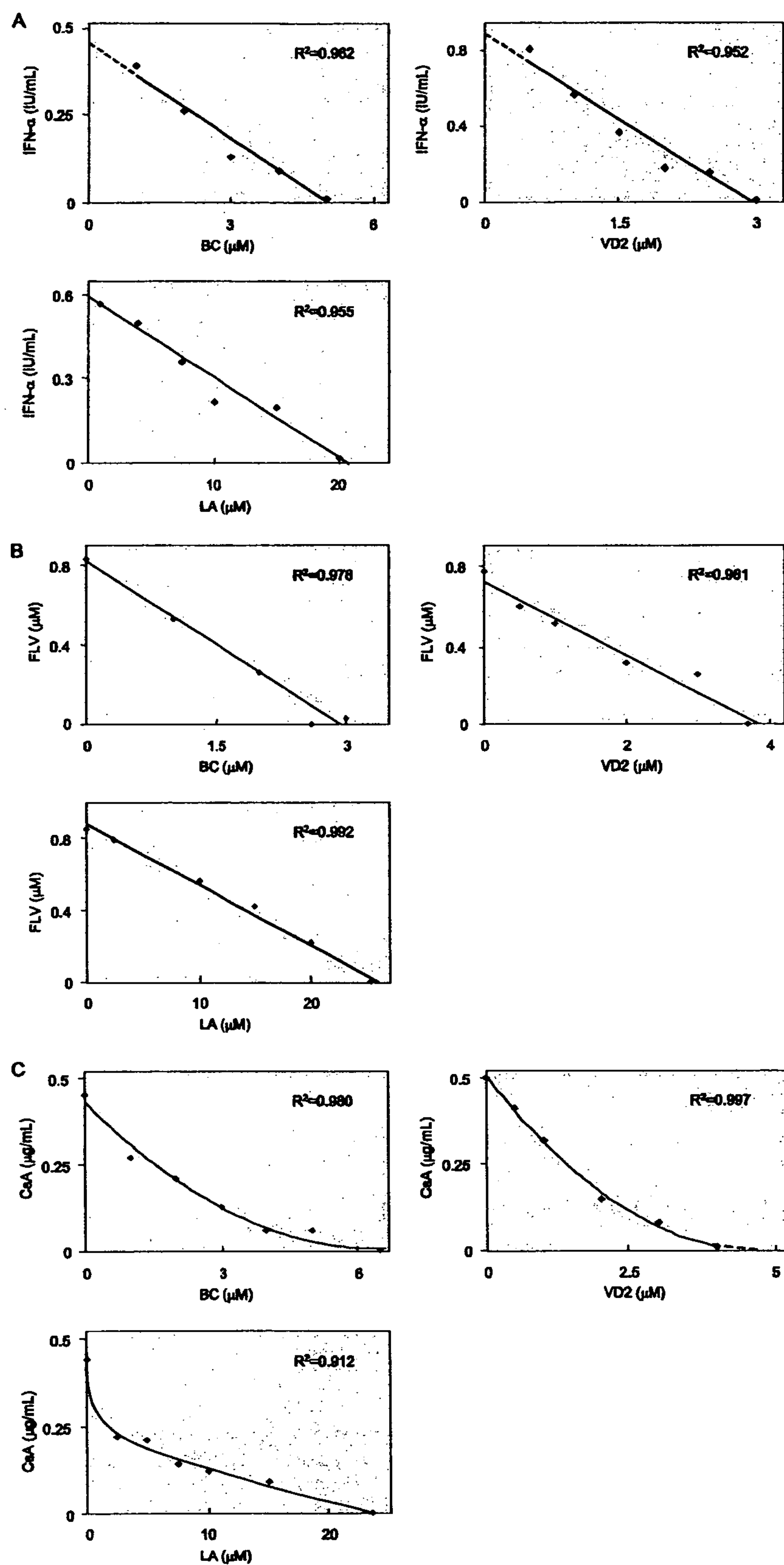

FIG. 7. Additive inhibitory effects of each of three nutrients in combination with IFN-α or FLV on HCV RNA replication, and synergistic effects observed with Cs. (A to C) Isobole plots of 50% inhibition of HCV RNA replication. OR6 cells were treated with BC (0, 1, 2, 3, 4, and 5 μM), VD2 (0, 0.5, 1, 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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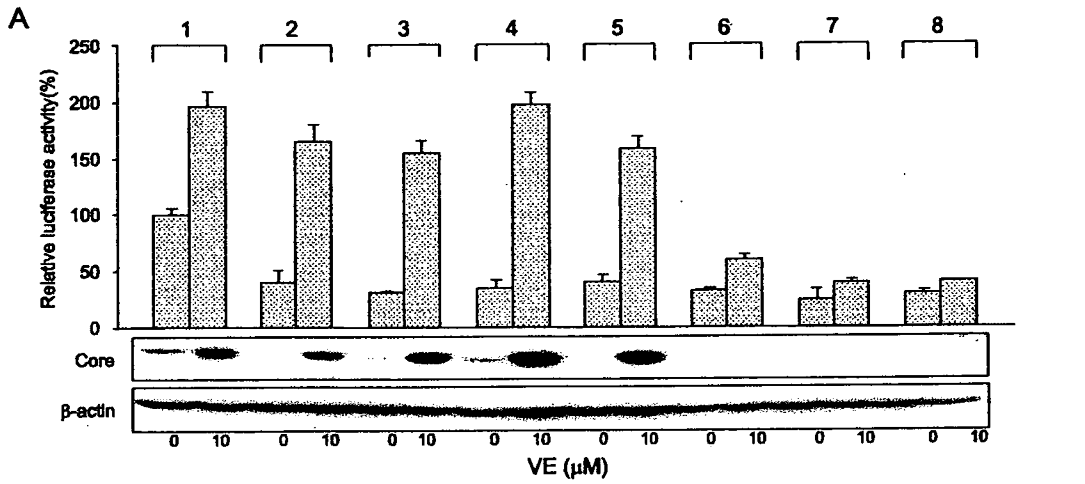

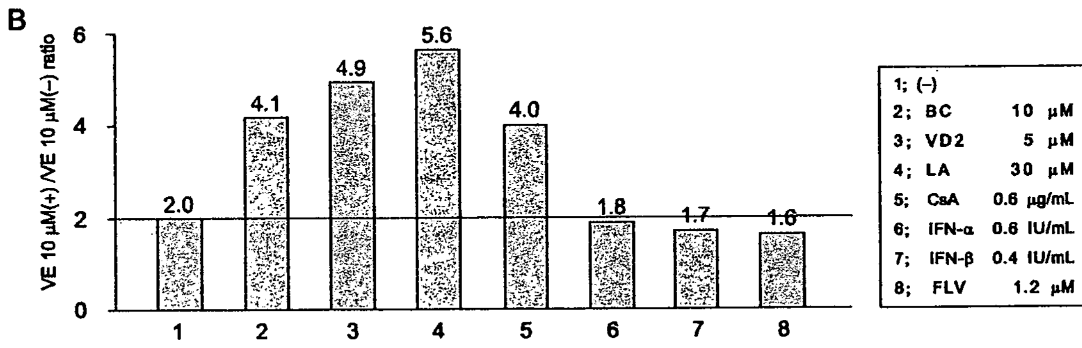

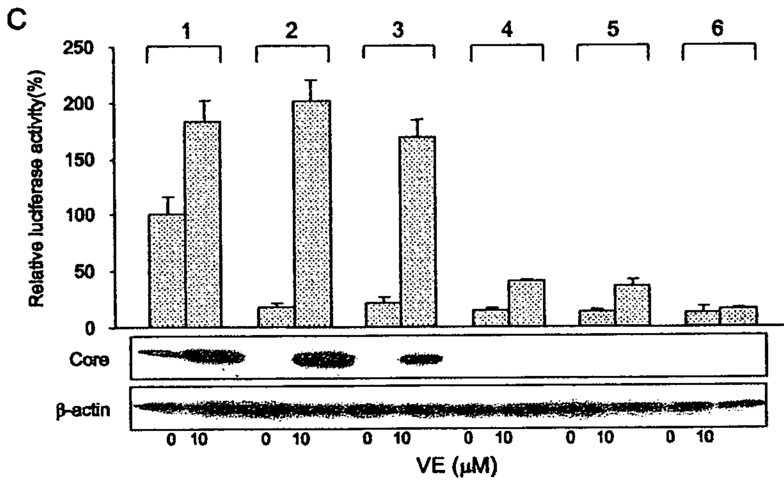

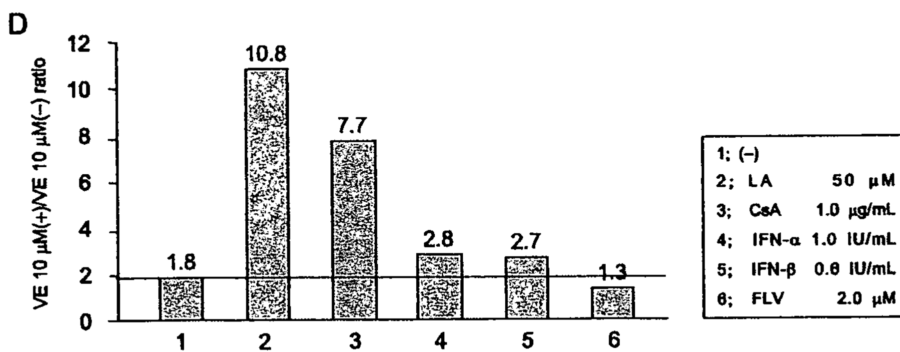

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isoboles in combinations using each nutrient and IFN-α or FLV graphed nearly straight lines in each case, indicating that the suppressive effects of these cotreatments on HCV RNA replication were additive (Fig. 7A and B). Similar additive effects were 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.

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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).

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.

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Serum-free cell culture system supplemented with lipid-rich albumin for hepatitis C virus (strain O of genotype 1b) replication

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Abstract

HuH-7 is a highly differentiated hepatoma cell line and the only cell line that supports robust RNA replication of the hepatitis C virus (HCV). HuH-7 cells cause cell death in serum-free culture condition. However, the effect is reversed by supplementation with selenium. Serum-free cell cultures are advantageous for vaccine development and experimental reproducibility. However, HCV RNA replication in HuH-7 cells in serum-free medium had not yet been achieved. Therefore, we tried to develop a system for robust HCV RNA replication in a serum-free cell culture. Although HuH-7 cells grew in serum-free medium in the presence of selenium, HuH-7 cells under these conditions did not support HCV RNA replication in long-term culture. Among the supplements tested, serum-free medium with lipid-rich albumin (LRA) was found to yield robust HCV RNA replication. HCV proteins were detected for more than 9 months in serum-free medium supplemented with LRA. This is the first report to demonstrate a long-term, serum-free cell culture that successfully maintained robust HCV RNA replication. This cell culture system is expected to be a useful tool for vaccine development, as well as for further investigation of cellular factors that are essential for HCV RNA replication.
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Keywords: HCV; Serum-free cell culture; Selenium; Lipid-rich albumin; Vaccine

1. Introduction

Persistent hepatitis C virus (HCV) infection causes liver fibrosis and hepatocellular carcinoma. Approximately 170 million people worldwide are infected with HCV. The combination of pegylated interferon (IFN) with ribavirin is the current standard therapy for chronic hepatitis C and yields a sustained virological response rate of about 55% (Feld and Hoofnagle, 2005). HCV, a member of the *Flaviviridae* family, is an enveloped virus with a positive single-stranded 9.6 kilobase (kb) RNA genome, which encodes a large polyprotein precursor of about 3000 amino acids (aa) (Kato et al., 1990; Tanaka et al., 1996). This polyprotein is processed by a combination of host and viral proteases into at least 10 proteins: Core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (Hijikata et al., 1991, 1993).

The discovery of the HCV subgenomic replicon in 1999 was a turning point for HCV RNA replication in cultured cells (Lohmann et al., 1999). Furthermore, genome-length HCV RNA replication systems were developed using N, Con1, and H strains (Blight et al., 2002; Ikeda et al., 2002; Pietschmann et al., 2002). We recently developed a genome-length HCV RNA (strain O of genotype 1b) replication reporter system (OR6) as an effective screening system (Ikeda et al., 2005). The development of infectious virus-producing cells has been a remarkable breakthrough in the fields of virology (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005).

HuH-7 is a highly differentiated hepatoma cell line that is commonly used for replication and infection studies. However, these cells have been reported by a number of groups to be heterogeneous, and the replication efficiency of this cell line differed among subclonal HuH-7 cells. Parental HuH-7 cells showed low capacity for HCV RNA replication and low susceptibility for HCV infection, but Huh7.5, Huh-Lunet, and our recently developed the RSc cells efficiently support HCV RNA replication and infection (Blight et al., 2002; Pietschmann et al., 2006; Ikeda et al., in preparation). In addition to these sub-

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clonal HuH-7 cell features, fetal bovine serum (FBS) may be another factor that affects HCV RNA replication and infection, as FBS is a pooled material containing unknown factors from different origins. Therefore, different FBS lots may affect the reproducibility of experiments conducted by different research groups. Furthermore, pathogens contained in FBS may introduce additional problems during the development of an HCV vaccine.

To resolve these issues, in this study, we investigated whether or not HCV RNA could replicate in serum-free cell culture. As HuH-7 cells produce an autocrine growth factor, hepatoma-derived growth factor, additional supplementation with growth factor seemed to be unnecessary (Nakamura et al., 1989, 1994). HuH-7 cells cause cell death in serum-free culture condition. However, when HuH-7 cells were cultured in serum-free medium supplemented with selenium, they produce a number of plasma proteins and liver-specific enzymes essential for their survival (Nakabayashi et al., 1982, 1984). Therefore, the serum-free culture of HuH-7 cells can be maintained by the addition of selenium alone. However, HCV RNA replication was not yet maintainable under these conditions.

In the present study, we found that HCV RNA replicates robustly for more than 9 months in serum-free medium supplemented with selenium and lipid-rich albumin (LRA). These results indicate the requirement of the lipid for HCV RNA replication. This cell culture system is expected to be a useful tool for the development of an HCV vaccine, and will also enhance the reproducibility of experiments, including those that evaluate anti-HCV reagents.

2. Materials and methods

2.1. Reagents

Sodium selenite (Na_2SeO_3), insulin, linoleic acid, oleic acid, IFN-α, and cyclosporine A (CsA) were purchased from Sigma–Aldrich (St. Louis, MO). Fluvastatin (FLV) and low-density lipoprotein (LDL) were purchased from Calbiochem (San Diego, CA). Lipid-rich albumin (ALBUMAX I™) was purchased from Invitrogen and is referred to as LRA in this study.

2.2. Cell cultures

The OR6 cells were cultured in Dulbecco's modified eagle's medium (DMEM; Gibco-BRL, Invitrogen Life Technology, Carlsbad, CA) supplemented with 10% FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1% penicillin–streptomycin (referred to as 10% FBS medium in this study), and G418 (300 μg per ml; Geneticin, Invitrogen) in a 5% CO_2 atmosphere at 37 °C. The cells were supplied with fresh medium twice a week at a 5:1 split ratio. The serum-free medium was DMEM containing 100 nM sodium selenite (Na_2SeO_3, Sigma–Aldrich) with LRA. The cells were cultured on six-well plates in 10% FBS medium or the serum-free medium. The cells cultured in the serum-free medium were harvested at 1, 3, 6, 9, and 12 months, and were subjected to Western blot analysis.

2.3. Cell count

To examine cell growth in selenium-containing medium with 10% FBS, 2 mg per ml of LRA, or no supplementation, OR6 cells were seeded at a density of 1×10^5 cells per well onto six-well plates in the absence of G418. Then, the number of the cells was counted in an improved Neubauer-type hemotocytometer after trypan blue dye (Invitrogen) treatment.

2.4. Western blot analysis

Preparation of the cell lysate, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblot analysis with a polyvinylidene difluoride membrane were performed as described previously (Kato et al., 2003). The antibodies used in this study were those against Core (Institute of Immunology, Tokyo, Japan), NS3 (Novocastra Laboratories, UK) and β-actin (AC-15; Sigma–Aldrich). Immunocomplexes were detected with the Renaissance enhanced chemiluminescence assay (Perkin-Elmer Life Sciences, Boston, MA).

2.5. Luciferase reporter assay

A luciferase reporter assay was performed as described previously (Ikeda et al., 2006). Briefly, 2×10^4 cells were plated onto 24-well plates and cultured in 10% FBS or the serum-free medium, at least in triplicate for each assay, and the cells were cultured for 24 h. Then, the cells were treated with human IFN-α, CsA, or FLV at several concentrations for 72 h. The cells were then harvested and subjected to luciferase assay using the *Renilla* luciferase assay system (Promega). The cells were washed twice with phosphate-buffered saline and were then extracted with 100 μl of *Renilla* lysis reagent. The relative luciferase unit value in 10 μl of lysates was measured by adding 50 μl of *Renilla* luciferase assay reagent according to the manufacturer's protocol. A manual Lumat LB 9501/16 luminometer (EG&G Berthold, Bad Wildbad, Germany) was used for the detection of luciferase activity.

3. Results

3.1. Efficiency of HCV RNA replication in HuH-7 cells with supplements in serum-free medium

At the early stage of the establishment of the HuH-7 cells, the serum-free cell culture was examined; the HuH-7 cells were found to replicate continuously for more than 9 months in a chemically defined medium containing selenium (Nakabayashi et al., 1982). Furthermore, the HuH-7 cells were maintained for a period of more than 3 years in improved serum-free medium containing additional supplements, i.e., oleic acid, linoleic acid, and insulin (Nakabayashi et al., 1984). We first investigated whether these serum-free conditions would support HCV RNA replication using the OR6 reporter system. The OR6 cells supported the replication of genome-length HCV-O RNA, into which the luciferase gene had been introduced (Ikeda et al., 2005). Using this OR6 system, we were able to monitor the level of HCV RNA

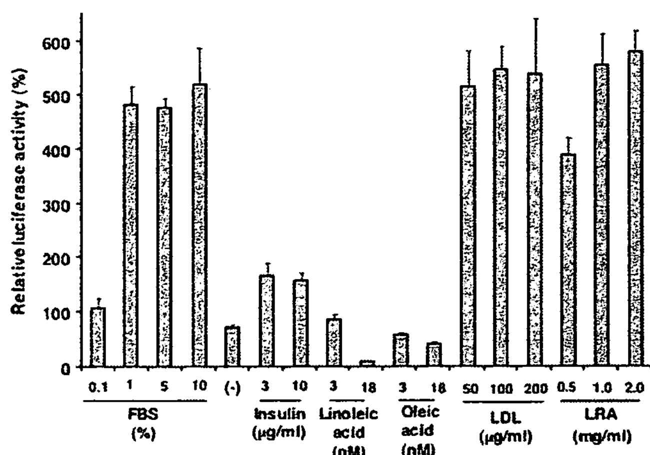

Fig. 1. HCV RNA replication in OR6 cells under different cell culture conditions. The OR6 cells were cultured in DMEM containing 100 nM sodium selenium with FBS (0.1, 1, 5, and 10%), insulin (3 and 10 µg per ml), linoleic acid (3 and 18 nM), oleic acid (3 and 18 nM), LDL (50, 100, and 200 µg per ml), or LRA (0.5, 1.0, and 2.0 mg per ml). The cells were harvested at 24 and 96 h and were subjected to luciferase assay as described in Section 2. Relative luciferase activities (%) were obtained from the value at 96 h, when the value at 24 h was assigned as 100%. The data indicate means ± standard deviations (S.D.s) from three independent experiments. (−) indicates culture in DMEM containing 100 nM sodium selenium.

replication by measuring the activity of luciferase. Luciferase activity at 96 h was five times higher than that at 24 h in 10% FBS medium (Fig. 1). However, HCV RNA replication was reduced when the OR6 cells were cultured in serum-free medium containing only selenium (Fig. 1). Serum-free medium supplemented with insulin reduced HCV RNA replication to about one-third of that observed in cultures maintained in 10% FBS medium supplemented with selenium. When used in combination with linoleic acid or oleic acid, the serum-free medium with selenium remarkably reduced HCV RNA replication (Fig. 1). However, in the serum-free medium with selenium in combination with LDL (50, 100, or 200 µg per ml) or LRA (1 or 2 mg per ml), HCV RNA replication was supported at the same level as that in 10% FBS medium, although the replication of HCV RNA was slightly low level in serum-free medium with selenium and LRA supplement at 0.5 mg per ml. These results suggest that chemically conditioned serum-free medium supplemented with selenium is not sufficient to support HCV RNA replication, but the addition of either LDL or LRA restored HCV RNA replication to almost the same level as that observed in 10% FBS medium. Thus, some of the elements essential for HCV RNA replication may be contained in LDL and LRA.

3.2. Cell growth of HuH-7 cells in selenium-containing medium supplemented with FBS or LRA

As HCV RNA replication depends on cell growth (Guo et al., 2001; Pietschmann et al., 2001), we next determined the number of cells at 24, 48, 72, and 96 h of culture. The doubling time of the OR6 cells was estimated to be approximately 29, 43, and 64 h in selenium-containing medium with 10% FBS, or 0.5, 1.0, and 2 mg per ml of LRA, or no supplementation, respectively

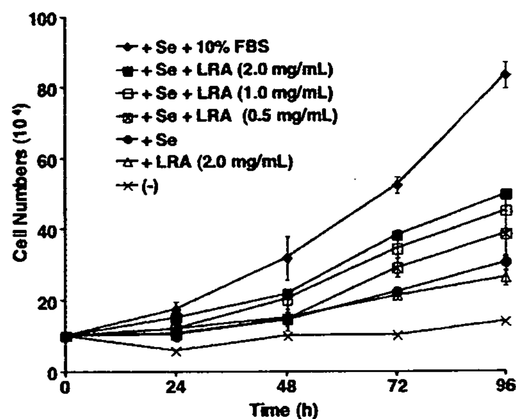

Fig. 2. Cell growth of HuH-7 cells in serum-free medium with LRA or in 10% FBS medium. The OR6 cells were plated at 1×10^5 cells per well onto six-well plates in triplicate. The cells were cultured in DMEM containing 100 nM sodium selenium with 10% FBS or 0.5, 1.0 and 2.0 mg per ml LRA or no supplement and were harvested at 24, 48, 72, and 96 h. The cells were cultured in DMEM or DMEM with LRA in the absence of selenium and were harvested at indicated time points.

(Fig. 2). Selenium or LRA containing medium enhanced the growth of OR6 cells and the combination of the selenium with LRA further enhanced the cell growth in a LRA dose-dependent manner (Fig. 2). Interestingly, the cell culture in serum-free medium supplemented with 2 mg per ml of LRA and selenium supported HCV RNA replication as efficiently as did that supplemented with 10% FBS and selenium (Fig. 1), although the cell growth of the culture in the medium with LRA and selenium was slower than that in the medium supplemented with 10% FBS and selenium. These results indicate that LRA may contain factors that enhance HCV RNA replication, and these LRA-derived factors appear to function in a manner that is not dependent on the cell growth factor.

3.3. Expression of HCV proteins in HuH-7 cells at 1 month of cell culture under various medium conditions

We continued to maintain the culture of OR6 cells for 1 month in different types of conditioned media. NS3 and Core HCV proteins were detected in the OR6 cell culture for 1 month in medium containing selenium with 10% or 5% FBS, but not with 1% or 0.5% FBS (Fig. 3A). The protein expression levels were higher in the cell culture with 10% FBS medium than in that with the 5% FBS medium. HCV proteins were not detected in the OR6 cells cultured in serum-free medium containing selenium alone (Fig. 3A). In contrast, HCV proteins were detected in LRA- and selenium-containing cell cultures. The levels of expression of HCV proteins were almost equal to those in the cell culture with selenium and 5% FBS (Fig. 3A). To further confirm the results, we performed luciferase assay for the OR6 cells cultured for 1 month after RNA transfection (Fig. 3B). These results indicated that HCV RNA replication was not maintained for more than 1 month in low concentrations (less than 1%) of FBS with selenium. However, the cell culture in serum-free medium with selenium and LRA at concentrations of 0.5, 1, or 2 mg per ml did efficiently support HCV RNA replication for more than

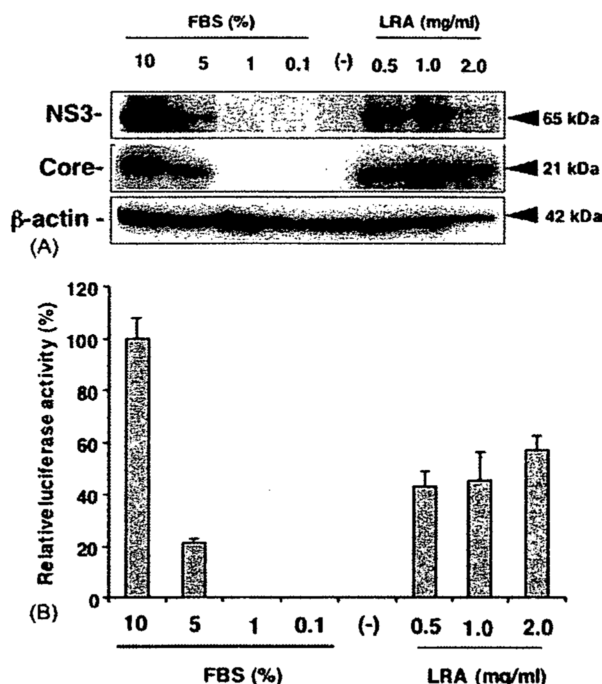

Fig. 3. Expression of HCV proteins in HuH-7 cells cultured in serum-free medium with LRA or in 10% FBS medium. OR6 cells were cultured for 30 days in DMEM containing 100 nM sodium selenium with LRA (0.5, 1.0, and 2.0 mg per ml) or FBS (0.1, 1, 5, and 10%). (−) indicates the culture in DMEM with sodium selenium. (A) The production of Core and NS3 in the OR6 cells was analyzed by immunoblotting using anti-Core and anti-NS3 antibodies. β-actin was used as a control for the amount of protein loaded per lane. (B) Relative luciferase activity was determined for the cells from 24-well plates in triplicate.

1 month. Therefore, we concluded that LRA could serve as an alternative supplement to FBS, when used in combination with selenium for HCV RNA replication.

3.4. Core expression in OR6 cells cultured in serum-free medium supplemented with LRA and selenium

Since OR6 cells cultured in serum-free medium with selenium and LRA could support HCV RNA replication at least for 1 month, we continued to culture the OR6 cells under the same conditions for 1 year. Core was detected for 9 months and for 6 months in serum-free medium containing selenium with LRA at 1.0 and 2.0 mg per ml, respectively (Fig. 4). These results suggest that serum-free cell culture supplemented with LRA supports HCV RNA replication at almost equal level to that in the culture supplemented with 10% FBS without G418 selection at least more than 9 months.

3.5. Influence of anti-HCV compounds on HCV RNA replication in OR6 cells cultured in serum-free medium supplemented with LRA and selenium

IFN is currently used as a therapeutic treatment of patients with chronic hepatitis C. Subgenomic HCV replicon-harboring cells and genome-length HCV RNA-replicating cells have been used to evaluate IFN and other candidate anti-HCV reagents, as

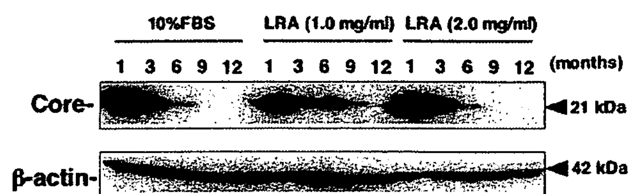

Fig. 4. Core expression in a long-term culture of OR6 cells in serum-free medium supplemented with LRA. The OR6 cells were cultured for 1 year in DMEM containing 100 nM sodium selenium with LRA at 1.0 or 2.0 mg per ml. The OR6 cells were also cultured in DMEM with 10% FBS and used as a positive control. Cells were harvested at 1, 3, 6, 9, and 12 months, and were subjected to Western blot analysis for Core using anti-Core antibody. β-actin was used as a control for the amount of protein loaded per lane.

has recently been reported in the case of CsA and the statins (Ikeda et al., 2006; Kapadia and Chisari, 2005; Watashi et al., 2003; Ye et al., 2003). However, one remaining problem with the evaluation of the anti-HCV activity of these reagents has been that FBS is derived from pooled blood materials and contains unknown cellular factors in various concentrations. These unknown factors can affect experimental results and reproducibility. Therefore, it is preferable to use a culture medium containing as few cellular factors as possible in order to ensure the reliability of the results. In this context, completely chemical conditioned medium is most ideal. Although our selenium and LRA culture system still contained animal proteins and lipids, it contained fewer unknown factors than medium containing FBS. We compared the anti-HCV activity of IFN-α, CsA, and FLV in serum-free medium with selenium and LRA, as well as in 10% FBS medium with selenium. OR6 cells were treated with these reagents for 72 h, and harvested cells were subjected to luciferase assay. When the OR6 cells were treated with IFN-α under either the 10% FBS medium condition or the selenium and LRA condition, HCV RNA replication was inhibited by IFN-α in a dose-dependent manner (range: 0.625–5 IU per ml; Fig. 5). However, the sensitivity of the cultures to IFN-α was greater in selenium and LRA medium than in 10% FBS medium (Fig. 5). When the OR6 cells were treated with CsA at low concentrations of 0.0625 and 0.125 μg per ml, sensitivity was greater in selenium and LRA medium than in 10% FBS medium, but almost identical sensitivity was observed under both medium conditions at concentrations of CsA of 0.25 and 0.5 μg per ml (Fig. 5). The most striking result was observed when the OR6 cells were treated with FLV in concentrations ranging from 1.25 to 10 μM. In contrast to the results obtained with CsA and IFN-α, the sensitivity associated with FLV was markedly reduced in the cultures treated with selenium and LRA medium, as compared to that of cultures in 10% FBS medium (Fig. 5).

4. Discussion

The development of serum-free cell culture systems will reduce the risk of contamination by infectious pathogens of animal origin in studies of vaccine development. Moreover, such systems will enhance the reproducibility of such experiments, because FBS contains unknown cellular factors that can affect the physiological state of cells. In this study, we developed a

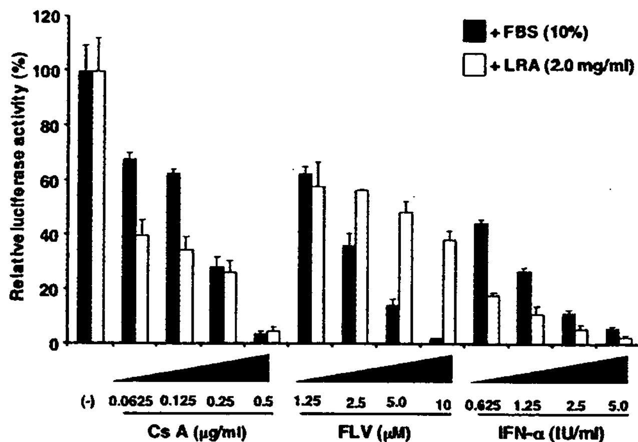

Fig. 5. Influence of anti-HCV compounds on HCV RNA replication in OR6 cells in serum-free medium supplemented with LRA or in 10% FBS medium. The OR6 cells were cultured in triplicate in 10% FBS medium with selenium (100 nM) or in serum-free medium with LRA (2 mg per ml) and selenium (100 nM). Under these cell culture conditions, the cells were treated with CsA (0.0625, 0.125, 0.25, and 0.5 μg per ml), FLV (1.25, 2.5, 5.0, and 10 μM), or IFN-α (0.625, 1.25, 2.5, and 5 IU per ml) for 72 h. Then, the cells were harvested and subjected to luciferase assay as described in Section 2. The luciferase activity of OR6 cells cultured under both medium conditions in the absence of anti-HCV compounds was assigned as 100% and is indicated by (−).

novel serum-free cell culture system supplemented with LRA that was able to support HCV RNA replication for more than 9 months. Although this cell culture system still contained animal proteins, the quantity of unknown cellular factors contained in the FBS was to a great extent reduced. The development of such a long-term cell culture is noteworthy, as it could be used for the stable mass-production of an HCV vaccine.

In a related previous study, it was reported that insulin, linoleic acid, and oleic acid enhance the growth of HuH-7 cells in serum-free cell culture (Nakabayashi et al., 1984). Therefore, we tested these supplements in HCV RNA replication experiments. Insulin was found to slightly enhance HCV RNA replication, but linoleic acid and oleic acid inhibited HCV RNA replication. In another recent study, Kapadia and co-workers reported that oleic acid enhanced HCV RNA replication in a serum-containing cell culture (Kapadia and Chisari, 2005). This discrepancy may have been due to the presence or absence of serum; moreover, some of the serum proteins may function in concert with oleic acid to support HCV RNA replication.

LDL is an exogenous source of cholesterol, and it was found to support HCV RNA replication in serum-free cell culture. When the cholesterol demand is satisfied, intrinsic mevalonate, which is a precursor of both cholesterol and non-sterol isoprenoid, is directed to non-sterol isoprenoid. Non-sterol isoprenoid is essential for the prenylation of cellular proteins that support HCV RNA replication (Ikeda et al., 2006; Kapadia and Chisari, 2005; Ye et al., 2003). For this reason, LDL may support HCV RNA replication, even in serum-free cell culture. LRA was initially developed to reduce or replace the requirement of serum supplementation by chromatographic separation from bovine plasma (Invitrogen). Here, we found that LRA supported HCV RNA replication as well as cell growth in serum-free cell culture. LRA contains free fatty acids and cholesterol associated with albumin. Therefore, cholesterol may, at least to some extent, play a role in HCV RNA replication by the mechanism described above. However, care should be taken before coming to a conclusion regarding the effects of free fatty acids on HCV RNA replication, because LRA contains a mixture of fatty acids in different states of saturation. Recent reports have demonstrated that saturated fatty acids enhance HCV RNA replication, but polyunsaturated fatty acids inhibit HCV RNA replication (Kapadia and Chisari, 2005). In addition, these diverse effects of fatty acids on HCV RNA replication in serum-containing medium cannot be simply applied to serum-free culture systems, because oleic acid has been shown to exert different effects on HCV RNA replication under serum-containing and serum-free culture conditions. To clarify the roles played by fatty acids in this context, further studies will be needed.

Here, HCV RNA replication depended on the growth of HuH-7 cells, and it has previously been shown that expression levels of HCV proteins and RNA are low in confluent cells (Guo et al., 2001; Pietschmann et al., 2001). Therefore, we examined the time course of cell growth and found that cell growth in serum-free medium with LRA was slower than that in 10% FBS medium, although the replication levels of HCV RNA were similar under both culture conditions studied. As regards HCV RNA replication and cell growth, Windisch et al. reported that HCV RNA replication in HuH-6 cells was not dependent on cell growth (Windisch et al., 2005). They demonstrated that the expression of HCV proteins was not reduced, even when the HuH-6 cells became confluent. In serum-free culture supplemented with LRA, HCV RNA replication in HuH-7 cells proceeds in a manner independent of cell growth, as was previously observed in the case of replication in HuH-6 cells.

One disadvantage associated with the use of FBS-containing cultures in virology studies is the influence exerted by unknown serum proteins, because FBS is derived from the serum pool of a bovine population. To prevent discrepancies between experiments due to differences between FBS lots, it is desirable to include only the most simple components as possible in the culture media. To this end, serum-free cultures are preferable in terms of reproducibility. Along these lines, it is expected that the use of our serum-free culture system with LRA may lead to improvements in experimental conditions for experiments in cell biology, as our culture medium contained only very simple supplements: fatty acids, cholesterol, albumin, and selenium. We tested the anti-HCV reagents CsA, FLV, and IFN-α in our serum-free culture supplemented with LRA. CsA and IFN-α were found to inhibit HCV RNA replication more efficiently in serum-free medium with LRA than in 10% FBS medium. Surprisingly, FLV inhibited HCV RNA replication less effectively in serum-free medium supplemented with LRA than it did in 10% FBS medium. One explanation for these differences may be that only FLV is a lipid metabolism-related reagent, and therefore the anti-HCV effect appeared to be antagonized by LRA. To clarify this issue, further study will be needed.

The goal of a serum-free cell culture is to develop a cell culture system containing only compounds that are of non-animal origin. Recently, a serum-free cell culture for canine

pathogenic virus production was reported using Madin Darby canine kidney cells lacking animal protein (Mochizuki, 2006). In this system, soybean peptone was used for the serum-free culture without animal protein. Canine viruses were able to grow almost as efficiently in this serum-free medium as in serum-containing medium. This plant protein-containing culture system is of the second-highest quality in terms of controlling for animal-derived pathogens in vaccine development experiments. Assessments of this animal protein-free cell culture system in terms of its usefulness for HCV RNA replication are therefore warranted.

We found HuH-7 cells supported HCV RNA replication for more than 9 months in serum-free medium supplemented with LRA at 1.0 mg per ml and selenium at 100 nM. This is the first report to describe HCV RNA replication in a long-term, serum-free culture. Recently, an infectious virus-producing cell culture system was reported using genotype 2a strain JFH1 (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). We are currently examining infectious virus production in a serum-free cell culture using the JFH1 virus. Our serum-free cell culture system may provide the useful information to the vaccine development.

In conclusion, we have established a serum-free cell culture system supplemented with LRA for the purpose of achieving HCV RNA replication. HCV proteins were detected during this series of experiments for more than 9 months. The present system has enabled an ongoing study of the production of an infectious HCV virion. Our serum-free cell culture system will yield relevant information for vaccine development, sustains only a relatively low risk of pathogenic contamination as compared to that of previous systems, and is expected to improve the reproducibility of similar experiments in the future.

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Cell culture-adaptive NS3 mutations required for the robust replication of genome-length hepatitis C virus RNA

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Abstract

We recently established a genome-length HCV RNA-replicating cell line (O strain of genotype 1b; here called O cells) using cured cells derived from sO cells, in which HCV subgenomic replicon RNA with an adaptive NS5A mutation (S2200R) is replicated. Characterization of the O cells revealed a second adaptive NS3 mutation (K1609E) required for genome-length HCV RNA replication. To clarify the role of adaptive mutation in genome-length HCV RNA replication, we newly established one and three kinds of genome-length HCV RNA-replicating cell lines possessing the cell background of sO and O cells, respectively, and found additional adaptive NS3 mutations (Q1112R, P1115L, and E1202G) required for the robust replication of genome-length HCV RNA. We further found that specific combinations of adaptive NS3 mutations drastically enhanced HCV RNA replication, regardless of the cell lines examined. These findings suggest that specific viral factors may affect the replication level of genome-length HCV RNA.
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Keywords: Hepatitis C virus; Adaptive mutation; Genome-length HCV RNA replication; HCV RNA-replicating cell line

1. Introduction

Infection with the hepatitis C virus (HCV), of the family *Flaviviridae*, frequently causes chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma. Since more than 170 million individuals are estimated to be infected with HCV worldwide, this disease is a global health problem (Thomas, 2000). HCV is an enveloped virus with a positive single-stranded 9.6 kilobase (kb) RNA genome, which encodes a large polyprotein precursor of about 3000 amino acids (aa) (Kato et al., 1990; Tanaka et al., 1995). This polyprotein is processed by a combination of the host and viral proteases into at least 10 proteins: the core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (Hijikata et al., 1991, 1993). These HCV proteins function not only in virus replication but may also affect a variety of cellular functions, including gene expression, signal transduction, and apoptosis (Bartenschlager and Lohmann, 2000; Kato, 2001).

Although studies on the mechanism of HCV replication were for many years difficult due to the lack of efficient HCV proliferation in cell cultures (Kato and Shimotohno, 2000), such studies proliferated after the development of subgenomic HCV replicon (Con-1 of genotype 1b) that was capable of replication in human hepatoma (HuH-7) cells (Lohmann et al., 1999). The subgenomic replicon RNA is composed of the HCV 5′-untranslated region (UTR) fused to the first 12 aa of the core coding region, the neomycin phosphotransferase (Neo^R) gene as a selectable marker, and the HCV NS3-NS5B regions under the control of an encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES), followed by 3′-UTR. After the first replicon, several additional replicons derived from H77 (1a), N (1b), 1B-1 (1b), O (1b), and JFH-1 (2a) strains were developed, and tissue, genotype, and host ranges were also expanded (Ali et al., 2004; Blight et al., 2000; Date et al., 2004; Ikeda et al., 2002; Kato and Sugiyama et al., 2003; Kato and Date et al., 2003; Kishine et al., 2002; Zhu et al., 2003). Since intracellular replicon RNAs were easily detected by Northern blot analysis and the HCV proteins produced were detected by

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Western blot analysis, these cell culture replication systems became valuable tools for basic studies of HCV, such as studies for viral replication and drug development (Bartenschlager, 2002, 2005; Lindenbach and Rice, 2005). However, in attempts to examine what happens in HCV-infected hepatocytes, subgenomic HCV replicons were insufficient because they lacked the effects of HCV structural proteins. For this reason, five kinds of genome-length HCV RNA-replicating cell lines, derived from H77 (1a), N (1b), Con-1 (1b), O (1b), and JFH-1 (2a) strains, have been established to date (Ikeda et al., 2002, 2005; Blight et al., 2002; Lindenbach et al., 2005; Pietschmann et al., 2002; Wakita et al., 2005; Zhong et al., 2005). Regarding the JFH-1 strain, the infectious virus was efficiently produced in cell culture (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005).

Studies in the past few years using subgenomic HCV replicon systems have revealed that most replicons possess cell culture-adaptive mutations, which enhance the efficiency of RNA replication and arise during G418 selection. Although these mutations have been found in most NS regions, they cluster in three distinct areas: the N-terminus of the NS3 helicase, two distinct positions of NS4B, and the center of NS5A (Appel et al., 2005; Blight et al., 2000, 2002, 2003; Grobler et al., 2003; Ikeda et al., 2002, 2005; Kato and Sugiyama et al., 2003; Kishine et al., 2002; Krieger et al., 2001; Lohmann et al., 2001, 2003; Yi and Lemon, 2004). To date, however, little information is available on the adaptive mutations obtained from a genome-length HCV RNA replication system. On the other hand, highly permissive cells (cured cells) for efficient RNA replication were also obtained by the elimination of replicons from the G418-selected cells by interferon (IFN) treatment (Blight et al., 2002; Kato and Sugiyama et al., 2003; Lohmann et al., 2003). These reports suggest that both viral and cellular factors determine the efficiency of RNA replication.

The sO replicon (O strain) that we developed also possesses a unique adaptive mutation (S2200R) in the center of NS5A (Kato and Sugiyama et al., 2003), and we recently established a genome-length HCV RNA-replicating cell line (O cells) by the transfection of genome-length HCV RNA with S2200R mutation into sOc cured cells, which were created by eliminating sO replicon from sO cells by IFN treatment (Ikeda et al., 2005). Sequence and functional analyses of HCV RNAs obtained from the O cells found a second adaptive mutation (K1609E) in the C-terminus of the NS3 helicase. We further found that the Oc cells, which were created by eliminating HCV RNA from O cells by IFN treatment, possessed more overwhelming advantages than the sOc cells in the replication of genome-length HCV RNA, even though the O cells were derived from sO cells (Ikeda et al., 2005). These results suggest that a second adaptive mutation, such as K1609E, is required for the robust replication of genome-length HCV RNA, and that the cell backgrounds regarding the potentials of genome-length HCV RNA replication differ greatly between Oc and sOc cells.

To evaluate these ideas, we newly established four kinds of genome-length HCV RNA-replicating cells possessing the cell background of sO or O cells, and then we characterized the genetic mutations detected in the replicating HCV RNAs. Here, we report the findings of adaptive NS3 mutations required for the robust replication of genome-length HCV RNA, and the drastic enhancement of HCV RNA replication by the specific combination of these adaptive NS3 mutations.

2. Materials and methods

2.1. Cell culture

sOc and Oc cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum as described previously (Ikeda et al., 2005). Cells supporting genome-length HCV RNAs were cultured in the presence of G418 (300 μg/ml; Geneticin, Invitrogen, Carlsbad, CA) and passaged twice a week at a 5:1 split ratio. HCV RNA-replicating cells possess the G418-resistant phenotype because *Neo*^{R} was produced by the efficient replication of HCV RNA. Therefore, when HCV RNA is excluded from the cells or when its level is decreased, the cells are killed in the presence of G418.

2.2. Plasmid constructions

To introduce the mutations into the plasmid pON/C-5B (GenBank accession no. AB191333; Fig. 1; Ikeda et al., 2005), a PCR-based site-directed mutagenesis method was used. The *Spe*I–*Not*I fragment (corresponding to positions 3474-6159 of the HCV genome) and the *Not*I–*Kpn*I fragment (corresponding to positions 6159–9077 of the HCV genome) of pHCV-O (Ikeda et al., 2005) were subcloned into pBluescript II (Stratagene, La Jolla, CA), resulting in pBlue/34AB and pBlue/5AB, respectively. pBlue/34AB and pBlue/5AB were used as the templates for PCR-based site-directed mutagenesis. The introduced mutations were confirmed by the sequencing of the obtained plasmids. The *Spe*I–*Not*I and *Not*I–*Kpn*I fragments possessing the mutation(s) were each replaced with the corresponding region of pHCV-O. The pON/C-5B-possessing mutation or mutations were generated by replacing the *Eco*RI–*Spe*I fragment of the pHCV-O, into which one or more mutations were introduced.

To construct pOR/C-5B, the *Neo*^{R} gene was replaced with the *Renillia luciferase* (RL) gene at *Asc*I and *Pme*I sites in pON/C-5B.

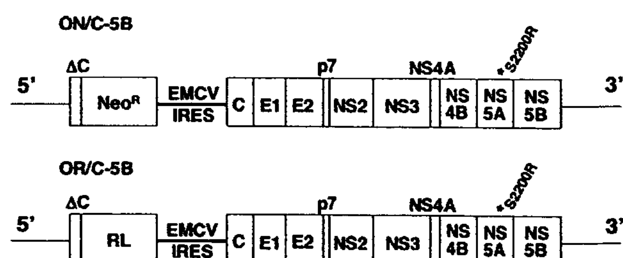

Fig. 1. Schematic gene organization of genome-length HCV RNAs used in this study. Open reading frames, untranslated regions, and EMCV IRES are depicted as open boxes, thin lines, and thick lines, respectively. ΔC indicates the 12N-terminal aa residues of the core as a part of IRES. ON/C-5B RNA and OR/C-5B RNA possess the *Neo*^{R} and RL genes, respectively. The asterisk indicates an adaptive mutation (S2200R) found in the sO subgenomic replicon (Kato and Sugiyama et al., 2003).

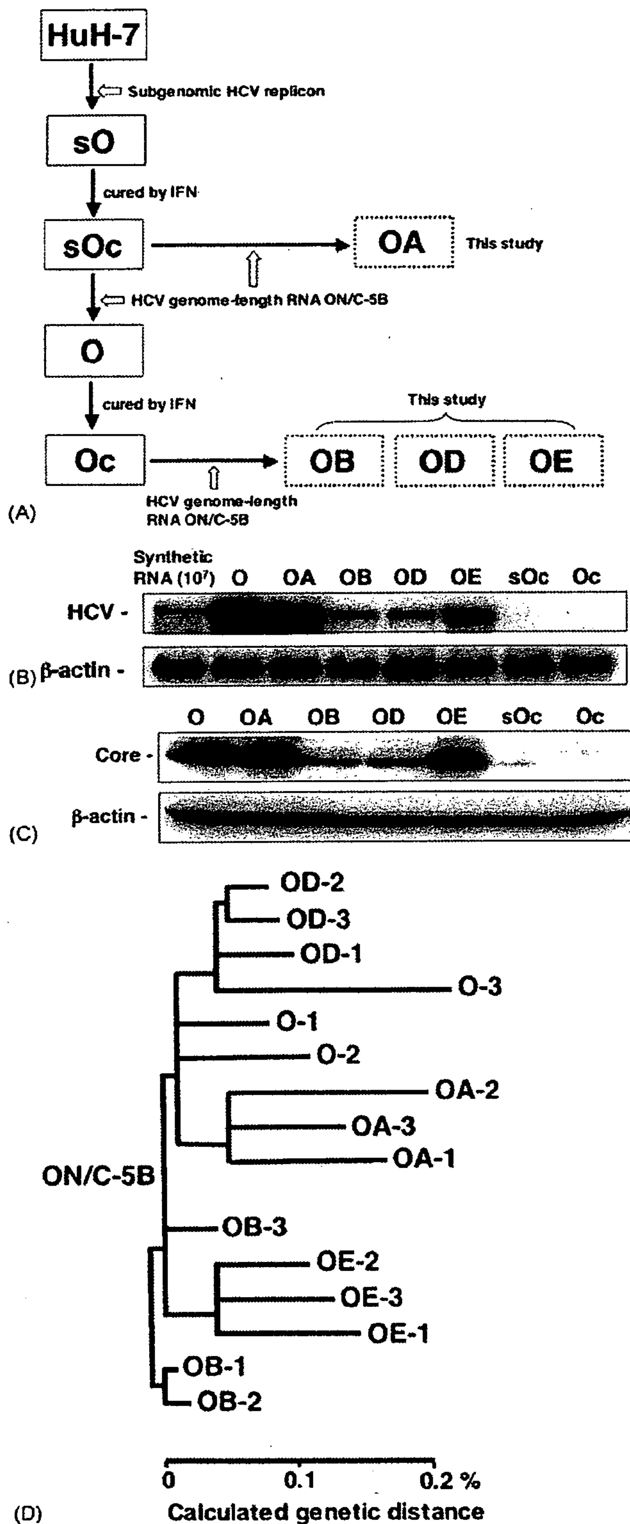

Fig. 2. Characterization of genome-length HCV RNA-replicating cell lines. (A) Lineage of genome-length HCV RNA-replicating cells. The sO and O cell lines were previously established (Ikeda et al., 2005; Kato and Sugiyama et al., 2003). (B) Northern blot analysis. Total RNAs from genome-length HCV RNA-replicating cells (O, OA, OB, OD, and OE cells), as well as total RNAs from the sOc and the Oc cells, were analyzed by Northern blot analysis using a positive-stranded HCV genome-specific RNA probe (upper panel) and a β-actin-specific probe (lower panel). In vitro-synthesized ON/C-5B RNA (10^7 genome equivalents spiked into normal cellular RNA) was used for the comparison of the expression level. (C) Western blot analysis. The orders of specimens were the same as in (B). Production of the core in these cells was analyzed by immunoblotting using anti-core antibody. β-actin was used as a control for the amount of protein loaded per lane. (D) Phylogenetic tree of HCV-O clone populations obtained from genome-length HCV RNA-replicating cells. The phylogenetic tree is depicted on the basis of nucleotide sequences of all clones obtained from the O, OA, OB, OD, and OE cells.

2.3. RNA synthesis

Plasmid DNAs were linearized with *Xba*I and used for RNA synthesis with the T7 MEGAscript kit (Ambion, Austin, TX). Synthesized RNA was purified by lithium chloride precipitation and dissolved in nuclease-free water.

2.4. RNA transfection and selection of G418-resistant cells

RNA transfection and selection of G418-resistant cells were carried out as described previously (Ikeda et al., 2005). Briefly, for electroporation, sOc or Oc cells were suspended at 10^7 cells/ml in phosphate-buffered saline (PBS), and then RNA was mixed with 500 μl of the cell suspension in a cuvette with a gap width of 0.2 cm (Bio-Rad Laboratories, Hercules, CA). The mixture was immediately subjected to two electric pulses of 1.2 kV, 25 μF, and maximum resistance. The cells were then seeded into a 10 cm diameter dish. After 24 h, the cells were selected in complete DMEM with 300 μg/ml G418 for 3 weeks.

2.5. Northern blot analysis

Total RNA from the cultured cells was prepared using an RNeasy extraction kit (Qiagen, Hilden, Germany). Three micrograms of RNA was used to detect the HCV RNA and β-actin mRNA. Northern blotting and hybridization were carried out as described previously (Ikeda et al., 2002; Kato and Sugiyama et al., 2003). A digoxigenin-labeled, negative-sense RNA probe complementary to the NS5B region (positions 8935–9374 of the HCV genome) and β-actin-specific antisense RNA probe were used to detect the HCV RNA and β-actin mRNA, respectively (Kato and Sugiyama et al., 2003; Kato et al., 2005).

2.6. Western blot analysis

The preparation of cell lysates, sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting analysis with a PVDV membrane were performed as described previously (Hijikata et al., 1993; Naganuma et al., 2000). The antibodies used in this study were those against Core (Institute of Immunology, Tokyo, Japan) and β-actin (AC-15; Sigma–Aldrich, St. Louis, MO). Immunocomplexes on the membranes were detected by enhanced chemiluminescence assay (Renaissance; Perkin–Elmer Life Sciences, Wellesley, MA).