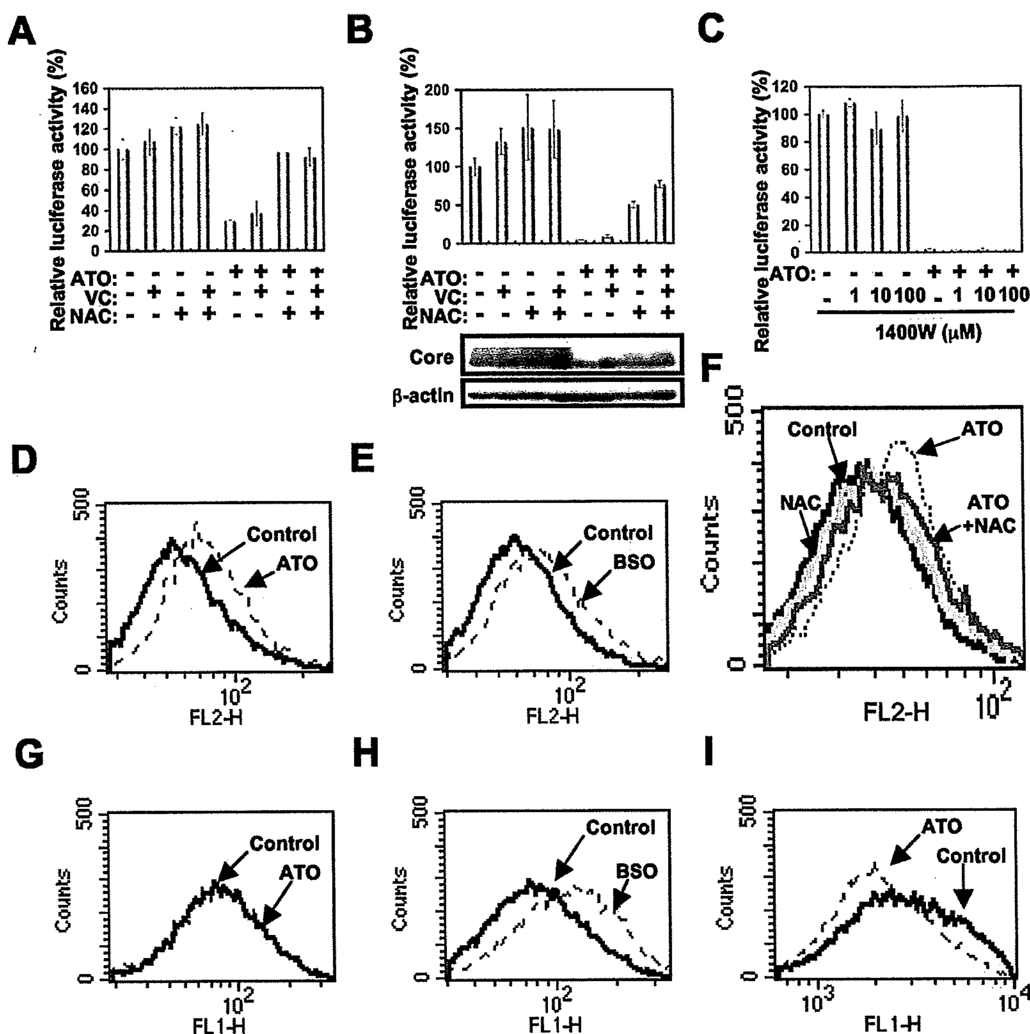

FIG. 6. The anti-HCV activity of ATO is associated with the glutathione redox system and oxidative stress. (A and B) The anti-HCV activity of ATO is eliminated by treatment with the antioxidant NAC. OR6 cells were treated with 1 μM ATO alone and in combination with 100 μM vitamin C (VC), with or without 10 mM NAC, for 24 h (A) or 72 h (B). The replication level of HCV RNA was monitored by the RL assay. The relative RL activity is shown. The results shown are means from three independent experiments; error bars indicate standard deviations. The results of Western blot analysis of cellular lysates with anti-HCV core or anti-β-actin antibody in OR6 cells at 72 h after the treatment with 1 μM ATO alone and in combination with 100 μM VC, with or without 10 mM NAC, are also shown. (C) Effect of combination treatment with ATO and the iNOS inhibitor 1400W on HCV RNA replication. OR6 cells were treated with 1 μM ATO alone and in combination with 1400W at the indicated concentrations for 72 h. The replication level of HCV RNA was monitored by the RL assay as described for panels A and B. (D and E) Effect of ATO on production of a ROS, O_2^-, in O cells. O cells were treated with 1 μM ATO (D) or 2 μM BSO (E) for 24 h. The intracellular O_2^- level was measured by flow cytometry using DHE as described in Materials and Methods. (F) Inhibition of ATO-dependent O_2^- induction by NAC. O cells were treated with either 1 μM ATO or 10 mM NAC alone and in combination with 10 mM NAC for 24 h. (G and H) Effect of ATO on production of a ROS, H_2O_2, in O cells. O cells were treated with 1 μM ATO (G) or 2 μM BSO (H) for 24 h. The intracellular H_2O_2 level was measured by flow cytometry using DCF as described in Materials and Methods. (I) Effect of ATO on the intracellular glutathione level in O cells. O cells were treated with 1 μM ATO for 72 h. The intracellular glutathione level was measured by flow cytometry using CellTracker Green CMFDA as described in Materials and Methods.

tively activate STAT3 and NF-κB, which are associated with HCV pathogenesis (19, 34, 36, 43, 49, 59, 60, 67). In fact, oxidative stress has been shown to trigger STAT3 tyrosine phosphorylation and nuclear translocation, which correlate with the activation of STAT3, leading to its DNA-binding activity (9). In contrast, ATO inhibited the STAT3 tyrosine phosphorylation through direct interaction with JAK kinase, thereby suppressing the transcriptional activity of STAT3 (12, 62). Importantly, STAT3 activation has been reported to be associated with HCV RNA replication (59, 69). The STAT3 Tyr705 dominant negative mutant has been shown to inhibit HCV RNA replication, suggesting that STAT3 positively regulates HCV replication (59). In contrast, others have reported that STAT3 induces anti-HCV activity (69). In this study, we analyzed the potential effect of ATO treatment on a set of stress-signaling events, including the NF-κB, AP-1, and STAT3 pathways, since ATO is known to modulate various signaling pathways. However, at 1 μM, which exerted an anti-HCV activity, the respective signaling pathways were not affected, arguing that the anti-HCV activity is independent of these

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pathways (Fig. 5). In this regard, these stress-signaling pathways have been reported to be constitutively activated in HCV core- or NS5A-expressing cells (19, 36, 49, 59, 60, 67). In addition, previous studies demonstrated that ATO modulates the NF-κB, AP-1, and STAT3 pathways at higher concentrations (NF-κB, >10 μM; AP-1, >30 μM; STAT3, >4 μM). Therefore, we may have only observed the marginal effect of ATO in this study (Fig. 5). On the other hand, the HCV core or NS3 protein as well as HCV infection induces NO, leading to induction of double-stranded DNA breaks and accumulation of mutations of cellular genes (35). However, the iNOS inhibitor 1400W could not suppress HCV RNA replication and the anti-HCV activity of ATO, indicating that NO is not associated with the anti-HCV activity or with HCV replication (Fig. 6C).

It has been indicated that oxidative damage plays an important role in the effect of ATO (38). ROS generated in response to ATO exposure lead to accumulation of intracellular H_2O_2. Glutathione peroxidase and catalase are key enzymes regulating the levels of ROS and protecting cells from ATO-induced damage (26). However, the gastrointestinal glutathione peroxidase was drastically downregulated in cells harboring HCV replicons, which are rendered more susceptible to oxidative stress (39). The glutathione redox system has been implicated in the cellular defense system (14, 20). Glutathione, a major antioxidant in cells, is a tripeptide synthesized from cysteine, glutamic acid, and glycine, and it can scavenge superoxide anion free radicals. ATO has been shown to bind to the sulfhydryl group of glutathione and deplete the intracellular glutathione, resulting in enhancement of the sensitivity to oxidative damage (20, 33). Conversely, the antioxidant NAC is readily taken up by cells and serves as a precursor to elevate intracellular glutathione (53). In fact, ATO-induced apoptosis has been shown to be inhibited by NAC (11, 14, 21, 28). In this study, we have demonstrated that the anti-HCV activity of ATO was completely eliminated by treatment with NAC for 24 h (Fig. 6A). In addition, we found that ATO increased intracellular O_2^- but not H_2O_2 and depleted the intracellular glutathione in HCV RNA-replicating cells (Fig. 6D to I). Importantly, NAC diminished the ATO-dependent O_2^- induction (Fig. 6F). This finding could strengthen the link between ATO-dependent oxidative stress and anti-HCV activity. Similarly, Wen et al. reported an increase in ROS and enhanced susceptibility to glutathione depletion in the HCV core-expressing HepG2 cells (61). Accordingly, ROS have been shown to significantly suppress RNA replication in HCV replicon-harboring cells treated with H_2O_2 (13). In addition, HCV replication has been shown to be inhibited by lipid peroxidation of arachidonate, and this peroxidation could be blocked by lipid-soluble antioxidants such as vitamin E (23). Conversely, several antioxidants, such as vitamin C, vitamin E, and NAC, enhanced HCV replication in the present study (Fig. 6A and B) (65). Thus, we suggest that ATO inhibited HCV RNA replication by modulating the glutathione redox system and oxidative stress. In contrast to the above findings with HCV, NAC has been shown to suppress HIV-1 replication by preventing the activation of HIV-1 long terminal repeat transcription by NF-κB, suggesting a correlation between a decrease in glutathione levels and activation of HIV-1 replication (46, 53, 54). In this context, ATO has shown opposite effects on HIV-1 and HCV replication, stimulating the former and inhibiting the latter. Considering all of these results together, ATO can be regarded as a useful, novel anti-HCV reagent. In addition, the host redox system may be critical for HCV replication and may represent a pivotal target for the clinical treatment of patients with chronic hepatitis C.

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Oxidative Stress Induces Anti-Hepatitis C Virus Status via the Activation of Extracellular Signal-Regulated Kinase

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Recently, we reported that β-carotene, vitamin D_2, and linoleic acid inhibited hepatitis C virus (HCV) RNA replication in hepatoma cells. Interestingly, in the course of the study, we found that the antioxidant vitamin E negated the anti-HCV activities of these nutrients. These results suggest that the oxidative stress caused by the three nutrients is involved in their anti-HCV activities. However, the molecular mechanism by which oxidative stress induces anti-HCV status remains unknown. Oxidative stress is also known to activate extracellular signal-regulated kinase (ERK). Therefore, we hypothesized that oxidative stress induces anti-HCV status via the mitogen activated protein kinase (MAPK)/ ERK kinase (MEK)–ERK1/2 signaling pathway. In this study, we found that the MEK1/2-specific inhibitor U0126 abolished the anti-HCV activities of the three nutrients in a dose-dependent manner. Moreover, U0126 significantly attenuated the anti-HCV activities of polyunsaturated fatty acids, interferon-γ, and cyclosporine A, but not statins. We further demonstrated that, with the exception of the statins, all of these anti-HCV nutrients and reagents actually induced activation of the MEK–ERK1/2 signaling pathway, which was inhibited or reduced by treatment not only with U0126 but also with vitamin E. We also demonstrated that phosphorylation of ERK1/2 by cyclosporine A was attenuated with *N*-acetylcysteine treatment and led to the negation of inhibition of HCV RNA replication. We propose that a cellular process that follows ERK1/2 phosphorylation and is specific to oxidative stimulation might lead to down-regulation of HCV RNA replication. *Conclusion:* Our results demonstrate the involvement of the MEK–ERK1/2 signaling pathway in the anti-HCV status induced by oxidative stress in a broad range of anti-HCV reagents. This intracellular modulation is expected to be a therapeutic target for the suppression of HCV RNA replication. (HEPATOLOGY 2009;50: 678-688.)

Abbreviations: AA, arachidonic acid; BC, β-carotene; CsA, cyclosporine A; CyPA, cyclophilin A; DHA, docosahexaenoic acid; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor: EPA, eicosapentaenoic acid; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; FLV, fluvastatin; HCV, hepatitis C virus; IFN, interferon; LA, linoleic acid: MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; NS5A, nonstructural 5A; PTV, pitavastatin; PUFA, polyunsaturated fatty acid; RL, renilla luciferase; ROS, reactive oxygen species; VD2, vitamin D_2; VE, vitamin E.

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Hepatitis C virus (HCV), which belongs to the family Flaviviridae, is a single-stranded positive-sense RNA virus of approximately 9.6 kb.[1,2] Persistent infection with HCV causes chronic hepatitis, which often leads to liver cirrhosis and hepatocellular carcinoma.[3] Therefore, HCV infection is a major health problem worldwide. Interferon (IFN)-based therapies, including the combination of pegylated IFN with ribavirin, are the current standard strategies for chronic hepatitis, but their sustained virological response rates are unsatisfactory.[4,5] There is thus an urgent need for novel partners with IFN or more effective reagents that may improve the sustained virological response rate.

Following the development in 1999 of a cell culture system to support efficient HCV RNA replication,[6] numerous studies have identified reagents that inhibit HCV RNA replication and enhance the effect of IFN treatment.[7-9] Some of these reagents are already available for clinical use. Previously, we also developed a genome-length HCV RNA (strain O of genotype 1b) replication system (OR6) with Renilla luciferase (RL) as a reporter in hepatoma cell lines.[10] Using this OR6 assay system, we found that mizoribine,[11] as an immunosuppressant, and

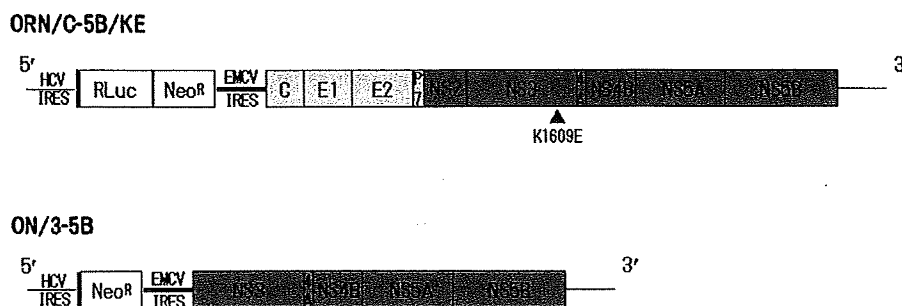

Fig. 1. Schematic gene organization of the genome-length and subgenomic HCV RNA used in this study. ORN/C-5B/KE encoding the RL gene was replicated in OR6 cells and ON/3-5B in sO cells. RL in OR6 cells was expressed as a fusion protein with neomycin phosphotransferase (Neo^R). The arrowhead indicates the position of K1609E, an adaptive mutation.

fluvastatin (FLV) and pitavastatin (PTV),[9,12] as the reagents for hypercholesterolemia, suppressed genome-length HCV RNA replication. Furthermore, in a recent study[13] in which we comprehensively analyzed the activities of ordinary nutrients on HCV RNA replication, three nutrients, β-carotene (BC), vitamin D_2 (VD2), and linoleic acid (LA), were found to suppress HCV RNA replication and enhance the antiviral activity of IFN-α or cyclosporine A (CsA) in an additive or a synergistic manner. Because the anti-HCV activities of these three nutrients, as well as CsA, were canceled by treatment with antioxidants such as vitamin E (VE) or selenium, we suggested that oxidative stress might be involved in the anti-HCV activities of these three nutrients and CsA. However, the detailed molecular mechanism via which the oxidative effects of these three nutrients and CsA suppress HCV RNA replication has not been explored.

The production of reactive oxygen species (ROS) plays a pivotal role in various cellular processes, including cell proliferation, differentiation, and apoptosis.[14] Whereas high-level production of ROS resulting from external stimuli is recognized as an important component of the pathogenesis of inflammatory and cancerous diseases, endogenously produced ROS at low concentrations are shown to function as signaling mediators of cellular responses.[15,16] Emerging evidence indicates that these ROS-triggered responses are mediated primarily via cellular signaling cascades, including a signaling pathway of extracellular signal-regulated kinase (ERK)1/2, namely p44/42 mitogen-activated protein kinase (MAPK), which belongs to the MAPK family.[17,18]

Several studies have revealed that certain viral proteins initiate activation of the MAPK/ERK kinase (MEK)–ERK1/2 signaling pathway, which may facilitate the viral replication and infectivity in the infected cells.[19,20] The HCV core protein[21] and the envelope protein[22] have also been reported to up-regulate this signaling pathway. However, another study reported that the HCV nonstructural 5A (NS5A) protein suppressed activating protein-1 activation by inhibiting the phosphorylation of ERK1/2 in replicon cells.[23] Moreover, recent studies using an inhibitor specific to the MEK–ERK1/2 signaling pathway reported that the direct anti-HCV activities of IFN-γ[24] and acetylsalicylic acid[25] are mediated in part through the induction of this cascade.

We demonstrate that the activation of MEK–ERK1/2 signaling plays a significant role in the anti-HCV activity caused by oxidative stress in a broad range of anti-HCV reagents.

Materials and Methods

Reagents and Antibodies. Dimethyl sulfoxide (DMSO), BC, VD2, VE, LA, arachidonic acid (AA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and IFN-γ were purchased from Sigma Aldrich (St. Louis, MO), and CsA, FLV, U0126, PD98059, SB203580, and c-Jun N-terminal kinase inhibitor II were obtained from Calbiochem (San Diego, CA). Epidermal growth factor (EGF) was purchased from Toyobo (Osaka, Japan). PTV was purchased from Kowa Company, Ltd. (Tokyo, Japan). Anti-HCV core antibody (CP11) was purchased from the Institute of Immunology (Tokyo, Japan), and anti-HCV NS5A antibody was the generous gift of Dr. A. Takamizawa (Research Foundation for Microbial Diseases, Osaka University). Antibodies specific to ERK1/2 (p44/42 MAPK), MEK1/2, and phosphorylated (S217/221) MEK1/2 were purchased from Cell Signaling Technology (Beverly, MA), and anti-phosphorylated (T202/Y204) ERK1/2 antibody was obtained from BD Biosciences (San Jose, CA). Anti–β-actin antibody was purchased from Sigma Aldrich.

Cell Cultures. The cell lines OR6 and sO were cloned from ORN/C-5B/KE RNA and subgenomic replicon RNA (ON/3-5B)–replicating cells, respectively (Fig. 1). These cells were derived from the hepatoma cell line HuH-7, cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), peni-

cillin, streptomycin, and 300 μg/mL of G418 (Geneticin; Invitrogen, Carlsbad, CA), and passaged twice a week at a 5:1 split ratio. ORN/C-5B/KE and ON/3-5B were derived from HCV-O (strain O of genotype 1b).[10]

OR6 Reporter Assay. For the RL assay, 1.0-1.5 $\times$ 10^4 OR6 cells were plated onto 24-well plates in triplicate and precultured for 24 hours. The cells were pretreated with DMSO or a specific inhibitor for 1 hour and then were treated with each anti-HCV nutrient or compound in either the absence (DMSO) or presence of a specific inhibitor for 72 hours. After the treatment, the cells were harvested with Renilla lysis reagent (Promega, Madison, WI) and subjected to RL assay according to the manufacturer's protocol.

Western Blot Analysis. For analysis of the effect of a specific inhibitor on the anti-HCV activity, 6.0-6.5 $\times$ 10^4 OR6 cells were plated onto 6-well plates and precultured for 24 hours. The pretreatment with DMSO or a specific inhibitor for 1 hour and subsequent treatment for 72 hours was performed in the same manner as for the OR6 reporter assay. For analysis of the activities of each anti-HCV nutrient or reagent on the MEK–ERK1/2 signaling pathway, 1.0 $\times$ 10^5 OR6 or sO cells were plated onto 6-well plates and precultured in 10% FBS-containing medium for 24 hours. After the preculture, the culture medium was changed to FBS-free medium and the cells were cultured for 48 hours prior to treatment with each nutrient or reagent. When the effect of a specific inhibitor or VE on ERK1/2 phosphorylation was analyzed, the cells were pretreated with the specific inhibitor or VE for 1 hour prior to each treatment. Preparation of the cell lysates, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting were then performed as described.[26]

Measurement of ROS. OR6 cells in 24-well plates were left untreated or were treated with hydrogen peroxide (1 mM), LA (200 μM), and CsA (15 μg/mL) for 30 minutes and then incubated with dihydrodichlorocarboxyfluorescein diacetate (Invitrogen) (5 μM) for 15 minutes. Fluorescence was measured with a FLUOROSKAN ASCENT fluorescence plate reader (Thermo Fisher Scientific, Waltham, MA) at an excitation wavelength of 485 nm and emission wavelength of 535 nm.

Cell Growth Assay. To examine the activity of EGF on OR6 cell growth, 6.0-6.5 $\times$ 10^4 OR6 cells were plated onto 6-well plates in triplicate and were pre-cultured for 24 hours. The cells were treated with or without EGF for 72 hours, and the number of viable cells was counted after trypan blue dye treatment as described.[11]

Statistical Analysis. Statistical comparison of the luciferase activities between the various treatment groups was performed using the Student t test. P values of less than 0.05 were considered statistically significant.

Results

Effects of MEK1/2-Specific Inhibitors on the Anti-HCV Activities of BC, VD2, and LA in OR6 Cells. Our recent study suggested the involvement of oxidative stress in the suppressive mechanism of three anti-HCV nutrients: BC, VD2, and LA.[13] Because there have been reports of negative regulation of HCV RNA replication via the MEK–ERK1/2 signaling pathway,[24,25] which is one of the oxidative stress-induced cellular signaling pathways, we hypothesized that the suppression of HCV RNA replication by these three nutrients might be mediated via this cascade (Supporting Fig. 1). To test this hypothesis, we first used an OR6 assay system to examine the effects of U0126 and PD98059, inhibitors specific to MEK1/2, on the three anti-HCV nutrients at 60% inhibitory concentration. As shown in Fig. 2A, treatment with either 5 μM of U0126 or 10 μM of PD98059 slightly enhanced HCV RNA replication in comparison with the control. However, U0126 attenuated the anti-HCV activities of the three nutrients more clearly than PD98059 (Fig. 2A,B). U0126 prevented the anti-HCV activities of the three nutrients in a significant and dose-dependent manner and exerted complete inhibition against the anti-HCV activities of BC and LA (Fig. 2C,D), while the inhibitory effect of PD98059 was more mild (Fig. 2E,F). As shown in Fig. 2G, we also found that U0126 treatment restored the expressions of HCV proteins, core, and NS5A in a dose-dependent manner. We further demonstrated that knockdown of MEK1 or MEK2 by small interfering RNA negated the anti-HCV activity of LA (Supporting Fig. 2A-C). These inhibitions by U0126 against the anti-HCV activities of the three nutrients were not due to the enhancement of encephalomyocarditis virus/internal ribosomal entry site–driven RL activity, because this activity was not increased by U0126 (data not shown). Moreover, treatment with neither SB203580 (an inhibitor specific to p38 MAPK) nor c-Jun N-terminal kinase inhibitor, both of which belong to the same cascade family as MEK–ERK1/2, significantly affected the anti-HCV activities of the three nutrients (data not shown). These results imply that the activation of the MEK–ERK1/2 signaling pathway might be required for the suppression of genome-length HCV RNA replication by the three nutrients in cell culture.

Effect of U0126 on the Suppressive Effects of Polyunsaturated Fatty Acids and Anti-HCV Reagents in OR6 Cells. Previous studies using a cell culture system have shown that polyunsaturated fatty acids (PUFAs), including LA, act as anti-HCV nutrients.[27,28] A recent study reported that lipid peroxidation of PUFAs was correlated with their anti-HCV activities, which were pre-

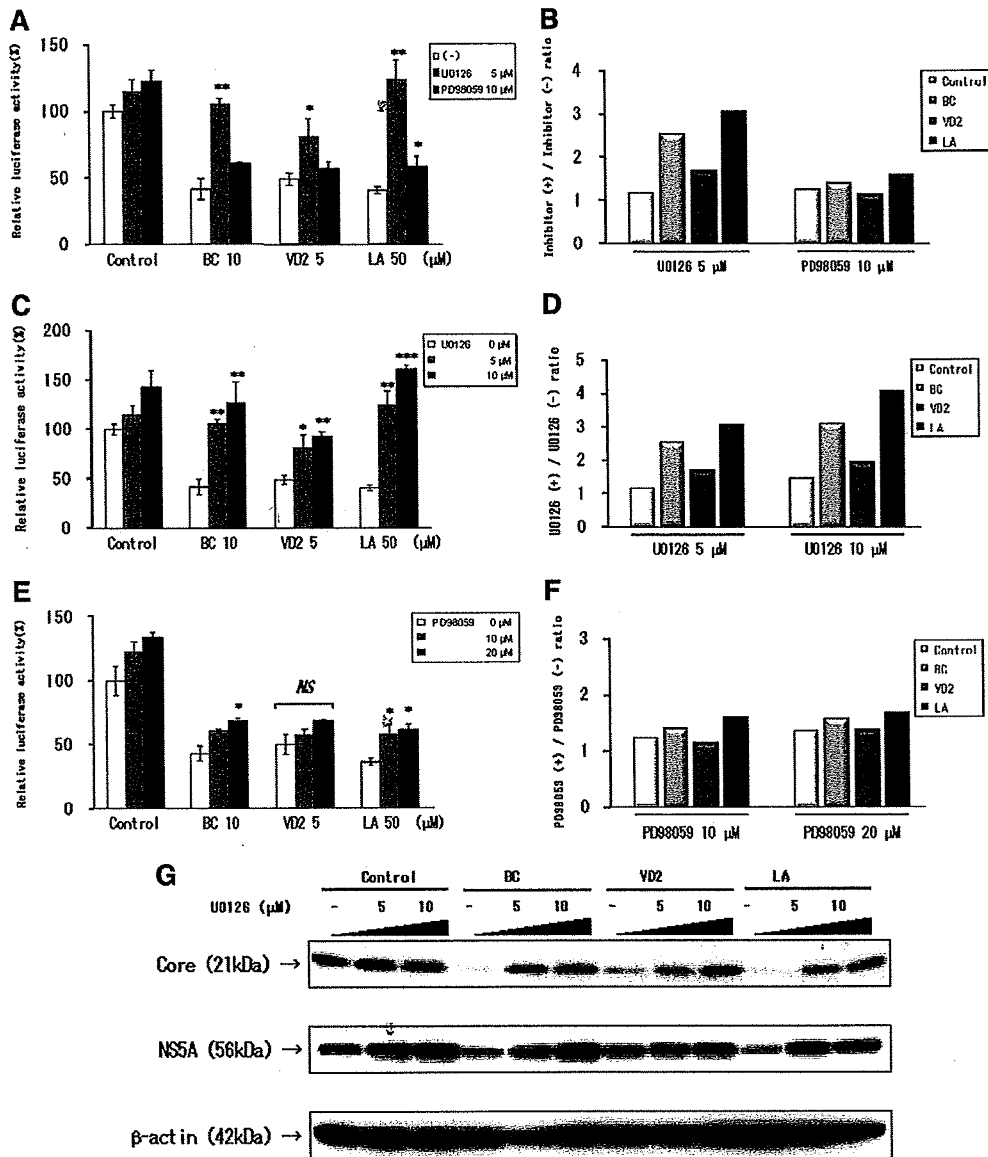

Fig. 2. U0126 strongly inhibited the anti-HCV activities of the anti-HCV nutrients BC, VD2, and LA in OR6 cells. (A,B) Effects of MEK-specific inhibitors on the three nutrients at the 60% inhibitory concentration. OR6 cells were pretreated with DMSO, 5 μM U0126, or 10 μM PD98059 for 1 hour. The cells were then treated with control medium, 10 μM BC, 5 μM VD2, or 50 μM LA in either the absence (DMSO) or presence of each specific inhibitor for 72 hours. After treatment, RL assay was performed as described in Materials and Methods. Shown here is the relative luciferase activity (%) calculated when the RL activity of the control was assigned as 100%. Data are expressed as the mean ± standard deviation of triplicate samples from at least three independent experiments. Asterisks indicate significant difference from treatment with DMSO (*$P < 0.05$; *$P < 0.01$) (A). The ratio of the RL activity in the presence of the MEK-specific inhibitor to the RL activity in the absence of the inhibitor was then calculated (B). (C-F) OR6 reporter assays of the dose effects of MEK1/2-specific inhibitors on the three nutrients. OR6 cells were pretreated with DMSO, U0126 (C), or PD98059 (E) at the indicated concentrations for 1 hour. Treatment of the cells with control medium or each of the three nutrients in either the absence (DMSO) or presence of each specific inhibitor and the RL assay of harvested OR6 cell samples were performed as described in panels A and B. Asterisks indicate significant difference from treatment with DMSO (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, not significant). Next, we calculated the ratio of RL activity in the presence of the MEK-specific inhibitor, U0126 (D), or PD98059 (F), to the RL activity in the absence of the inhibitor. (G) Western blot analysis of the dose effects of U0126 on three nutrients. OR6 cells were pretreated and then treated as in panel C. The production of HCV core and NS5A in the cells was analyzed by way of immunoblotting using antibodies specific to HCV core (top row) and NS5A (middle row). β-actin was used as a control for the amount of protein loaded per lane (bottom row).

vented by treatment with VE.[29] This result coincides with our previous observations on the effects of LA.[13] We proposed that the MEK–ERK1/2 signaling pathway might be involved in the anti-HCV activity of PUFAs, including LA, because lipid peroxidation is known to be a ROS-triggered cellular modification.[16] As expected, treatment with U0126 attenuated the anti-HCV activities of four representative PUFAs in a significant and dose-dependent manner (Fig. 3A,B).

Moreover, because the anti-HCV activities of BC, VD2, LA, and CsA, but not FLV, were found to be negated by VE,[13] we were also interested in the potent role of the MEK–ERK1/2 signaling pathway in the anti-HCV mechanism of CsA. Furthermore, the previous study using a subgenomic replicon system had already shown the partial involvement of this cascade in the antiviral activity of IFN-γ.[24] Therefore, we examined the effects of U0126 on various anti-HCV reagents: IFN-γ, CsA, and statins (FLV and PTV). We confirmed that also in genome-length HCV RNA replication cells, U0126 significantly inhibited the anti-HCV activity of IFN-γ (Fig. 3C,D). Interestingly, consistent with the effects of treatment with VE,[13] the anti-HCV activity of CsA was completely abrogated by U0126 in a significant and dose-dependent manner, whereas statins were unaffected (Fig. 3C,D).

U0126 restored the reduced expression of HCV proteins by PUFAs, IFN-γ, and CsA in a dose-dependent manner, whereas statins were unaffected (Fig. 3E,F). These results were supported by additional real-time reverse-transcription polymerase chain reaction and immunofluorescence analyses (Supporting Fig. 3A-C). We also observed that knockdown of MEK1 or MEK2 by small interfering RNA did not affect the anti-HCV activity of PTV (Supporting Fig. 2A-C). Collectively, these findings suggest that the MEK–ERK1/2 signaling pathway may play a critical role in the negative regulation of HCV RNA replication by the anti-HCV nutrients BC and VD2, PUFAs, and the anti-HCV reagents IFN-γ and CsA, but not statins.

Activation of the MEK–ERK1/2 Signaling Pathway by Anti-HCV Nutrients and Reagents. To further ensure the involvement of the MEK–ERK1/2 signaling pathway in the suppressive mechanisms of anti-HCV nutrients and reagents, we next examined whether these nutrients and reagents could actually initiate the activation of this signaling pathway. After treating the HCV RNA replicating cells with each of the nutrients and reagents, we performed immunoblotting specific to the phosphorylation of ERK1/2 and MEK1/2. In the same way as EGF, a potent activator of these kinases, the three anti-HCV nutrients (BC, VD2, and LA) enhanced the phosphorylation of ERK1/2 and MEK1/2 in both genome-length and subgenomic HCV RNA replication cells (Fig. 4A,B). IFN-γ, CsA, and all of the PUFAs also up-regulated this cascade in OR6 cells (Fig. 4C,D). The increase in phosphorylation of ERK1/2 was not observed after either statin treatment (Fig. 4D). The activation of MEK–ERK1/2 by the three anti-HCV nutrients was apparent until 1 hour after their application and subsequently attenuated, although EGF exhibited persistent enhancement of MEK–ERK1/2 phosphorylation (Fig. 4E). Because the experiments regarding ERK1/2 phosphorylation were performed in FBS-free conditions, we checked the anti-HCV activity of PTV, CsA, and LA in FBS-free medium. The results revealed that these anti-HCV reagents and nutrients also inhibited HCV RNA replication in FBS-free conditions (Supporting Fig. 4). Taken together, these findings indicate that the anti-HCV nutrients and reagents activated the MEK–ERK1/2 signaling pathway in HCV RNA replicating cells, providing further confirmation that this signaling cascade might be involved in their anti-HCV activities.

MEK1/2-Specific Inhibitors Attenuated the Increased Phosphorylation of ERK1/2 by Anti-HCV Nutrients/Reagents and EGF. We next tested whether MEK1/2-specific inhibitors could prevent not only the suppression of HCV RNA replication but also the activation of ERK1/2 by the anti-HCV nutrients BC, VD2, and PUFAs and the anti-HCV reagents IFN-γ and CsA. Consistent with the inhibitory effects on their anti-HCV activities, U0126 more markedly abrogated the increase in ERK1/2 phosphorylation by anti-HCV nutrients, reagents, and EGF than did PD98059 (Fig. 5A,B). As shown in Fig. 5C, the enhanced ERK1/2 phosphorylation by the three nutrients and EGF was reduced by U0126 in a dose-dependent manner.

VE Attenuated the Increased Phosphorylation of ERK1/2 by Anti-HCV Nutrients/Reagents and EGF. Because the suppression of HCV RNA replication by BC, VD2, LA, and CsA were completely negated by the treatment with VE in our recent study,[13] we investigated whether VE could also inhibit ERK1/2 activation by anti-HCV nutrients and reagents. As expected, VE also attenuated the enhanced phosphorylation of ERK1/2 by not only anti-HCV nutrients and CsA but also IFN-γ and EGF (Fig. 6A,B). We also demonstrated that phosphorylation of ERK1/2 by CsA was attenuated with *N*-acetylcysteine treatment and led to the negation of inhibition of HCV RNA replication (Supporting Fig. 5A-C). The anti-HCV nutrients and reagents, whose activities were negated by U0126, were also inhibited by VE. In contrast, the anti-HCV activities of statins were not negated by U0126 or VE. We also demonstrated that LA and CsA induce ROS (Fig.

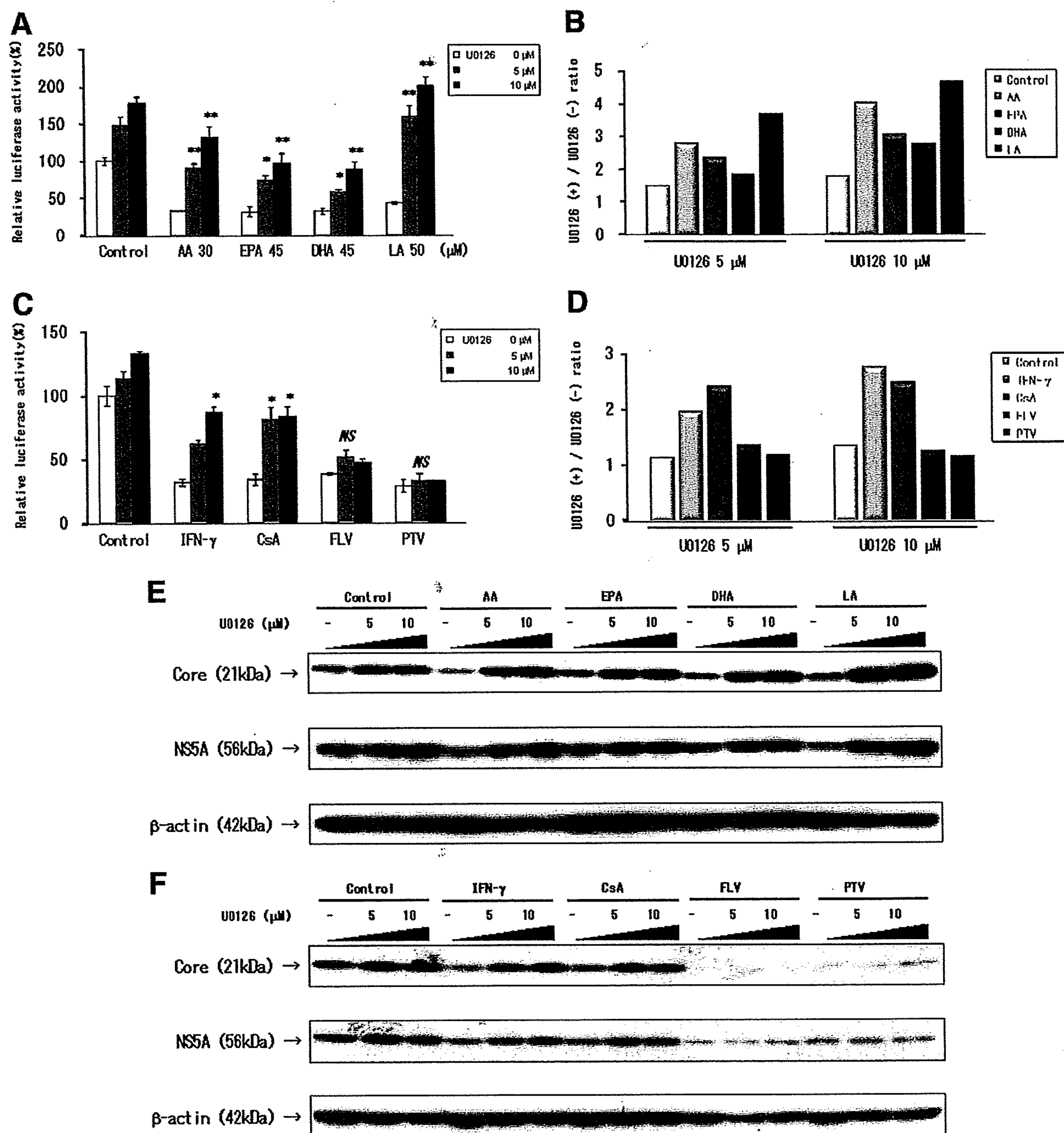

Fig. 3. U0126 dose-dependently attenuated the anti-HCV activities of PUFAs, IFN-γ, and CsA, but not the statins. (A-D) OR6 reporter assays of the dose effects of U0126 on the PUFAs and anti-HCV reagents at the 60% inhibitory concentration. OR6 cells were pretreated with DMSO or U0126 as in Fig. 2C and then treated with control medium, 30 μM AA, 45 μM EPA, 45 μM DHA, or 50 μM LA (A) and control medium, 0.4 IU/mL IFN-γ, 0.2 μg/mL CsA, 3 μM FLV, or 1 μM PTV (C), respectively, in either the absence (DMSO) or presence of U0126 for 72 hours. After the treatment, the RL assay of harvested OR6 cell samples was performed as described in Fig. 2A and 2B. Asterisks indicate significant difference from treatment with DMSO (*$P < 0.05$; **$P < 0.01$; NS, not significant). The ratio of the RL activity in the presence of U0126 to the RL activity in the absence of U0126 was then calculated (B, D). (E, F) Western blot analysis of the dose effects of U0126 on the PUFAs and anti-HCV reagents. The production of HCV core (top row) and NS5A (middle row) in the cells treated as in panel A (E) and panel C (F) was analyzed as described in Fig. 2G. β-actin was used as a control for the amount of protein loaded per lane (bottom row).

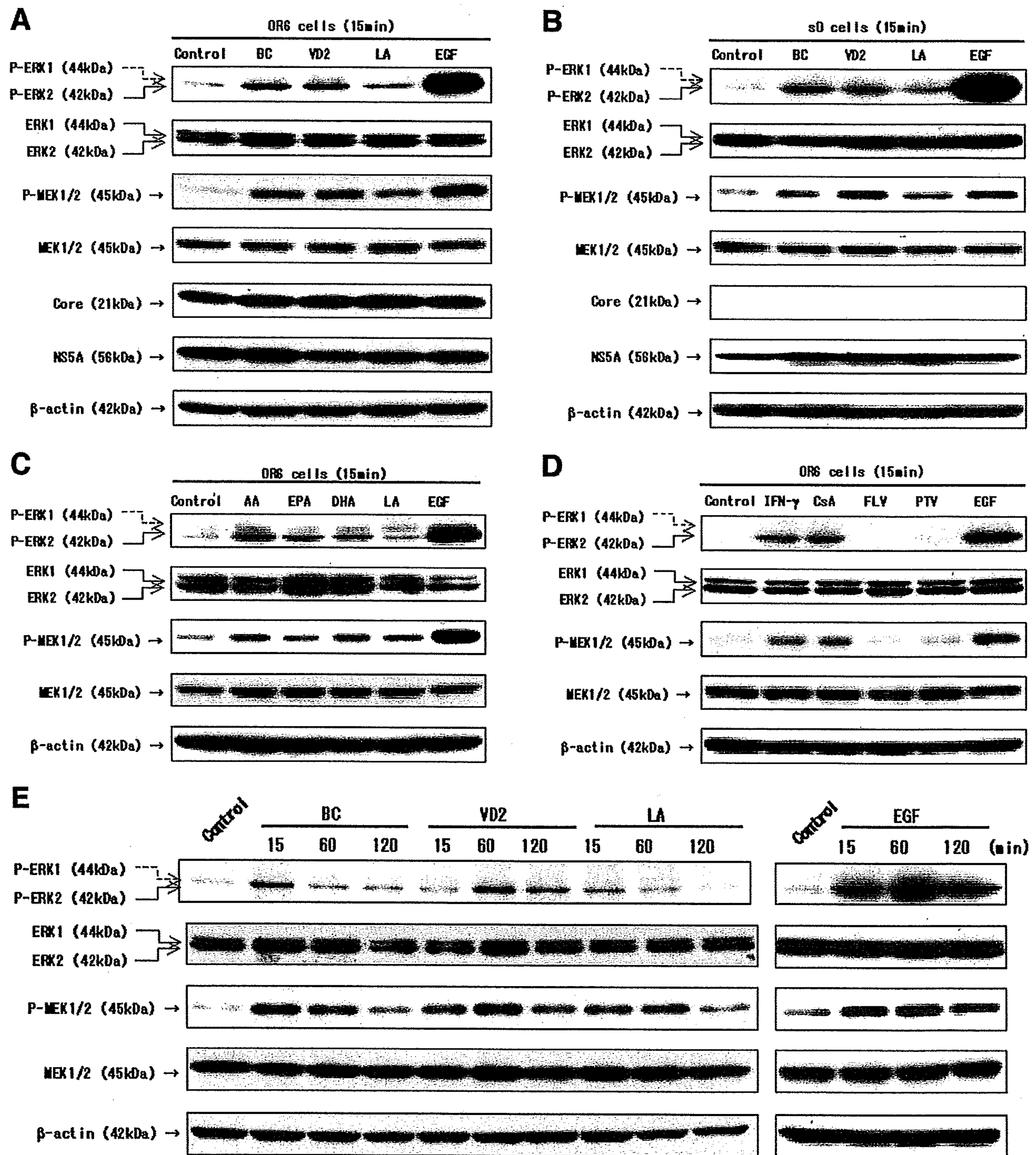

Fig. 4. U0126 attenuated the MEK-ERK1/2 signaling pathway activated by anti-HCV nutrients and reagents. (A, B) Three anti-HCV nutrients–BC, VD2, and LA–increased the phosphorylation of MEK-ERK1/2 in both full-length and subgenomic HCV RNA replication cells. OR6 cells (A) or sO cells (B) were maintained in FBS-free medium for 48 hours and then treated with control medium, 20 μM BC, 10 μM VD2, 100 μM LA, or 50 ng/mL EGF for 15 minutes. After treatment, cell lysates underwent western blot analysis using antibodies specific to phosphorylated ERK1/2, ERK1/2, phosphorylated MEK1/2, and MEK1/2. The appropriate expression of HCV core and NS5A was determined by way of immunoblotting with their respective antibodies. (C, D) IFN-γ, CsA, and the PUFAs, but not the statins, increased the phosphorylation of MEK-ERK1/2 in OR6 cells. OR6 cells were precultured as described in panels A and B, then treated with control medium, 100 μM AA, EPA, DHA, or LA, or 50 ng/mL EGF (C) and control medium, 2 IU/mL IFN-γ, 2 μg/mL CsA, 5 μM of FLV or PTV, or 50 ng/mL EGF (D), respectively, for 15 minutes. (E) Time-course western blot analysis of the increase of MEK-ERK1/2 phosphorylation by the three anti-HCV nutrients and EGF. Samples for analysis were harvested prior to treatment with the control medium, 20 μM BC, 10 μM VD2, 100 μM LA, or 50 ng/mL EGF (0 time point) and at 15, 60, and 120 minutes posttreatment. After all of the treatments (C-E), cell lysates were subjected to western blot analysis of the activation of the MEK-ERK1/2 signaling pathway as described in panels A and B. β-actin was used as a control for the amount of protein loaded per lane in all analyses.

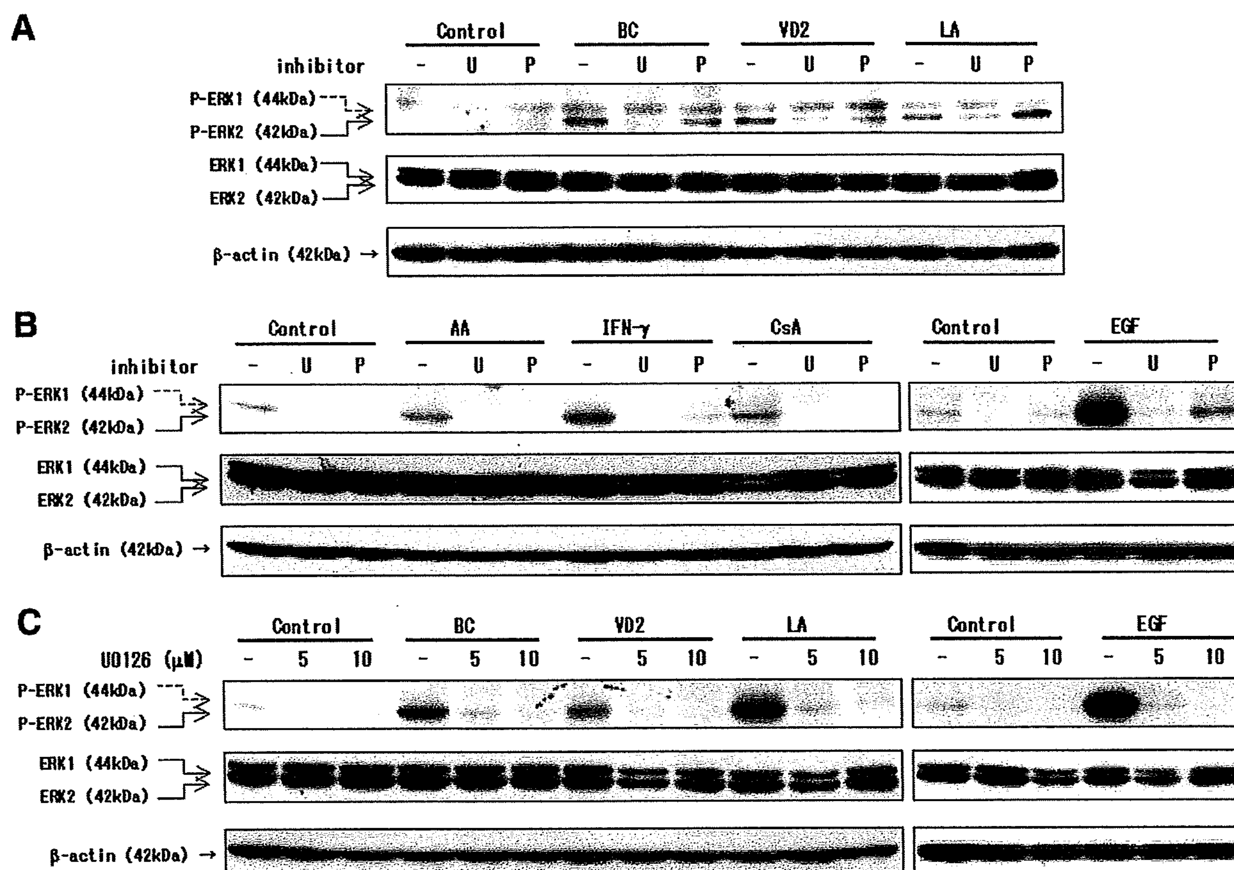

Fig. 5. U0126 strongly abolished ERK1/2 phosphorylation by the anti-HCV nutrients, anti-HCV reagents, and EGF. (A,B) Effects of the MEK1/2-specific inhibitors on ERK1/2 phosphorylation by anti-HCV nutrients and reagents. OR6 cells were precultured as described in Figs. 4A and B, and then pretreated with DMSO (−), 10 μM U0126: (U), or 20 μ M PD98059: (P) for 1 hour. Subsequently, the cells were treated with control medium, 20 μM BC, 10 μM VD2, or 100 μM LA (A) and control medium, 100 μM AA, 2 IU/mL IFN-γ, 2 μg/mL CsA, or 50 ng/mL EGF (B), respectively, in either the absence (DMSO) (−) or presence of U0126 (U) or PD98059 (P) for 15 minutes. (C) Dose effects of U0126 on ERK1/2 phosphorylation by the three anti-HCV nutrients and EGF. OR6 cells were precultured as described in Figs. 4A and 4B, then pretreated with DMSO (−) or 5 or 10 μM U0126 for 1 hour. The cells were then treated with control medium, 20 μM BC, 10 μM VD2, 100 μM LA, or 50 ng/mL EGF in either the absence (−) or presence of U0126 for 15 minutes. After all treatments (A-C), cell lysates were subjected to western blot analysis using antibodies specific to phosphorylated ERK1/2 (top row) and ERK1/2 (middle row). β-actin was used as a control for the amount of protein loaded per lane (bottom row).

7). Collectively, these results suggest that these nutrients and reagents induce ROS as an oxidant in HCV RNA replicating cells, leading to activation of the MEK–ERK1/2 signaling pathway and suppression of HCV RNA replication.

The Effects of EGF on HCV RNA Replication were Different than Those of the Anti-HCV Nutrients/Reagents. Because the study by Huang et al.[24] showed that EGF time-dependently suppressed the expressions of HCV nonstructural proteins in subgenomic replicon-harboring cells, we wondered whether EGF could suppress genome-length HCV RNA replication. EGF inhibited HCV RNA replication by approximately 25% at a concentration of 100 ng/mL. This anti-HCV activity was weaker than that of the anti-HCV nutrients and reagents tested in this study. However, as shown in the cell growth assay, EGF promoted OR6 cell proliferation in a dose-dependent manner (Supporting Fig. 6). These cell growth effects of EGF may have caused us to underestimate the actual anti-HCV activity of EGF. The other reagents and nutrients did not affect cell proliferation compared with EGF (Supporting Fig. 7).

Discussion

The previous studies using the MEK1/2-specific inhibitor and subgenomic replicon system showed that induction of the MEK–ERK1/2 signaling pathway might be required for the suppression of HCV RNA replication by some reagents.[24,25] In agreement with the study by Huang

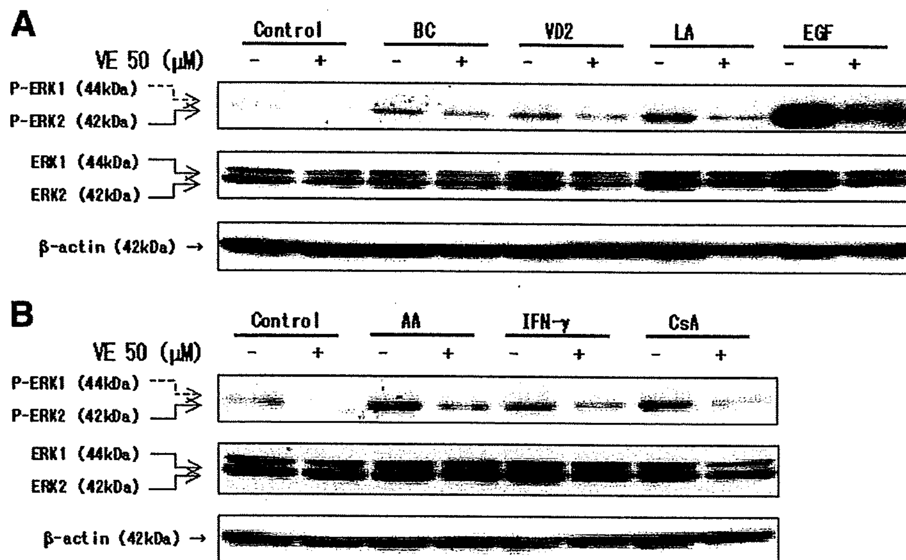

Fig. 6. VE attenuated ERK1/2 phosphorylation by the anti-HCV nutrients and reagents. OR6 cells were precultured as described in Figs. 4A and B, and then pretreated with ethanol (−) or 50 μM VE (+) for 1 hour. The cells were then treated with control medium, 20 μM BC, 10 μM VD2, 100 μM LA, or 50 ng/mL EGF (A) and control medium, 100 μM AA, 2 IU/mL IFN-γ, and 2 μg/mL CsA (B), respectively, in either the absence (ethanol) (−) or presence (+) of 50 μM VE for 15 minutes. After the treatment, cell lysates underwent western blot analysis as described in Fig. 5.

et al.,[24] we also confirmed that U0126 inhibited the anti-HCV activity of IFN-γ in OR6 cells stably replicating genome-length HCV RNA. Although they did not identify the direct activation of the MEK–ERK1/2 signaling pathway by IFN-γ, we demonstrated that IFN-γ could stimulate this cascade in HCV RNA replication cells. Moreover, this stimulation was not only inhibited by U0126 but also by antioxidant VE. This result indicates the involvement of oxidative stress in the anti-HCV activity of IFN-γ as well as the MEK–ERK1/2 signaling pathway. IFNs induce the transcription of IFN-stimulated genes through the JAK–STAT pathway, but the induction of IFN-stimulated genes by IFN-γ has been far more complex than that by IFN type I.[30] A study using a macrophage cell line revealed that IFN-γ activated ERK1/2, followed by the expression of IFN-γ–stimulated genes downstream of the JAK–STAT signaling pathway.[31] Another study reported that the defensive activity of IFN-γ against hepatitis B virus in hepatoblastoma cells was mediated through the induction of oxidative stress.[32] Furthermore, ROS itself has been reported to suppress HCV RNA replication in human hepatoma cells.[33] These reports support our proposal regarding anti-HCV activity of oxidative stress that the generation of intracellular ROS inhibits HCV RNA replication through activation of the MEK–ERK1/2 signaling pathway. Waris and Siddiqui[34] reported that calcium-dependent ROS generation induced cyclooxygenase-2 and prostaglandin E(2) via the activation of nuclear factor kappa B, leading to the suppression of HCV RNA replication. Choi et al.[35] also demonstrated that elevated calcium suppressed HCV RNA replication. The activation of nuclear factor kappa B by ROS was mediated through the MEK–ERK1/2 signaling pathway. Therefore, we suggest that the oxidative reagents and nutrients in this study also may induce anti-HCV status by calcium-dependent ROS generation.

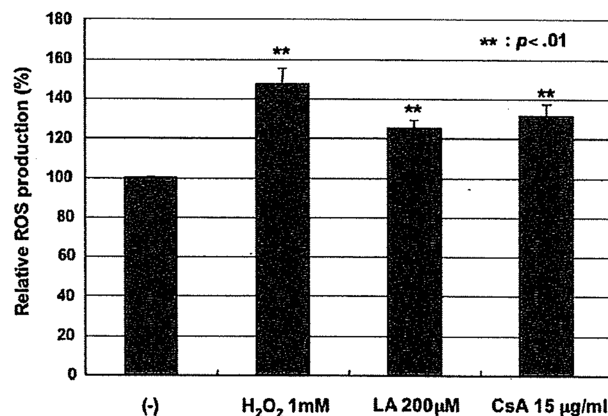

Fig. 7. ROS production by H_2O_2, LA, and CsA. OR6 cells were untreated or treated with H_2O_2 (1 mM), LA (200 μM), and CsA (15 μg/mL) and then incubated with dihydrodichlorocarboxyfluorescein diacetate. Fluorescence was measured with a fluorescence plate reader. $^{**}P < 0.01$ versus untreated cells.

In the course of our study of the anti-HCV activities of these three nutrients, we found that treatment with U0126 more strongly inhibited their anti-HCV activities than treatment with PD98059. U0126 has been shown to possess approximately 100-fold-higher MEK1/2-specific inhibitory activity than PD98059.[36] This different potential between the two inhibitors was considered to cause a gap in their effects on anti-HCV activities. We further found that, much like EGF, all three nutrients enhanced the phosphorylation of ERK1/2 and MEK1/2, which was reduced by treatment with U0126 or VE. In addition, the

present study was the first to observe that BC, which has been shown to produce ROS,[37] activates the MEK–ERK1/2 signaling pathway, an action that VD2[38] and LA[39] have already been shown to exhibit in leukemia cell and dendritic cell lines, respectively. Furthermore, we found the involvement of the MEK–ERK1/2 signaling pathway in the anti-HCV mechanism of the three nutrients as well as various PUFAs, which were reported to be mediated through lipid peroxidation.[29] These results suggest that the anti-HCV nutrients BC, VD2, and PUFAs, including LA, as well as IFN-γ may suppress HCV RNA replication via activation of the MEK–ERK1/2 signaling pathway in response to ROS production.

We also investigated the involvement of the MEK–ERK1/2 signaling pathway in the suppressive mechanism of anti-HCV reagents other than IFN-γ. In our previous study, the anti-HCV activity of CsA, but not FLV, was prevented by VE.[13] Consequently, these results implied that CsA, but not statins, could be potent activators of the MEK–ERK1/2 signaling pathway as oxidants, leading to down-regulation of HCV RNA replication. CsA has been demonstrated to bind to cyclophilins and suppress HCV RNA replication by abolishing their interaction with NS5B polymerase.[40] This CsA binding to cyclophilins, especially cyclophilin A (CyPA), has been shown to result in the generation of ROS through inhibition of the peptidylprolyl-cis-trans-isomerase–like activity of CyPA.[41] Moreover, CyPA was reported to be secreted in response to oxidative stress,[42] and to bind to a cell surface receptor, CD147, followed by ERK1/2 activation.[43] These reports and our results suggest that CsA, acting as an oxidant, may trigger activation of the MEK–ERK1/2 signaling pathway, both directly by producing ROS by way of interaction with CyPA in the early phase, and indirectly by secreting CyPA in the late phase. Both activations could lead to an inhibition of HCV RNA replication. Thus, CyPA may play a critical role as an intermediator in the oxidative anti-HCV activity of CsA. In the latest study, CyPA was identified as the most essential cellular cofactor of HCV RNA replication among cyclophilins.[44] Further studies will be needed to clarify whether CyPA is required for the oxidative suppressive mechanism of anti-HCV nutrients/reagents other than CsA.

Although we expected that strong activation of the MEK–ERK1/2 signaling pathway would suppress HCV RNA replication, EGF exhibited only slight anti-HCV activity in OR6 cells. The promotion of cell growth by EGF might prevent its primary inhibitory effect on HCV RNA replication. A portion of the ERK1/2 phosphorylation by EGF was also reduced by treatment with VE (Fig. 6A), suggesting that EGF might stimulate the MEK–ERK1/2 signaling pathway, in part, as an oxidant, and that this oxidative activity of EGF could exhibit its slight anti-HCV activity.

In this study, using MEK1/2 specific inhibitors, we revealed that the MEK–ERK1/2 signaling pathway is involved in the oxidative antiviral mechanism of the anti-HCV nutrients BC, VD2, and PUFAs and the anti-HCV reagents IFN-γ and CsA. Our results suggest that this oxidative induction of the MEK–ERK1/2 signaling pathway could be a novel therapeutic strategy for the eradication of HCV infection. Although oxidants themselves cause liver damage, they may work as anti-HCV factors during therapy in patients with chronic hepatitis C.

In conclusion, this study suggests that the anti-HCV activity of oxidative stress is closely linked to the activation of the MEK–ERK1/2 signaling pathway.

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Strain-Dependent Viral Dynamics and Virus-Cell Interactions in a Novel *In Vitro* System Supporting the Life Cycle of Blood-Borne Hepatitis C Virus

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We developed an *in vitro* system that can be used for the study of the life cycle of a wide variety of blood-borne hepatitis C viruses (HCV) from various patients using a three-dimensional hollow fiber culture system and an immortalized primary human hepatocyte (HuS-E/2) cell line. Unlike the conventional two-dimensional culture, this system not only enhanced the infectivity of blood-borne HCV but also supported its long-term proliferation and the production of infectious virus particles. Both sucrose gradient fractionation and electron microscopy examination showed that the produced virus-like particles are within a similar fraction and size range to those previously reported. Infection with different HCV strains showed strain-dependent different patterns of HCV proliferation and particle production. Fluctuation of virus proliferation and particle production was found during prolonged culture and was found to be associated with change in the major replicating virus strain. Induction of cellular apoptosis was only found when strains of HCV-2a genotype were used for infection. Interferon-alpha stimulation also varied among different strains of HCV-1b genotypes tested in this study. *Conclusion:* These results suggest that this *in vitro* infection system can reproduce strain-dependent events reflecting viral dynamics and virus-cell interactions at the early phase of blood-borne HCV infection, and that this system can allow the development of new anti-HCV strategies specific to various HCV strains. (HEPATOLOGY 2009;50:689-696.)

Hepatitis C virus (HCV) is a serious problem worldwide, with 3% of the world's population chronically infected.[1] Chronic infection with HCV may lead to high rates of liver cirrhosis and hepatocellular carcinoma.[2] Because the HCV standard therapy is still insufficient for treating many patients,[3] the development of more effective and less toxic anti-HCV agents is desired. The virological studies required to reach this goal need reproducible and efficient HCV proliferation in cell culture. An *in vitro* infection system using recombinant HCV-JFH1 was developed. In this system, HuH7 cells transfected with *in vitro*-synthesized JFH1-RNA were

Abbreviations: 2D, two-dimensional; 2D-HuS-E/2, HuS-E/2 cells cultured in two-dimensional condition; 3D, three-dimensional; 3D/HF, 3D hollow fibers; 3D-HuS-E/2, HuS-E/2 cells cultured in three-dimensional condition in the hollow fibers; HCV, hepatitis C virus; IFN-α, interferon alpha; LDH, lactate dehydrogenase; p.i., postinfection; RFB, radial-flow bioreactor; RT-PCR, reverse transcription polymerase chain reaction.

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shown to secrete infectious viral particles.[4] This system, however, requires the combination of HuH-7-derived cell lines and JFH1-based constructs, limiting its usefulness for studying other HCV strains. Because HuH-7 cells cannot support the complete life cycle of blood-borne HCV (bbHCV) derived from clinical samples,[5] this system is insufficient for studying all the events related to bbHCV infection.

Many researchers have attempted to develop an *in vitro* system for bbHCV.[6-8] These current systems, however, are still insufficient due to their low efficiency for infectivity and replication of bbHCV. Working toward this same goal, we recently established immortalized primary human hepatocyte cell lines by transducing them with E6 and E7 genes from the human papilloma virus 18.[5,9] As expected, we observed improved infection and replication of bbHCV especially in one of these cell lines (HuS-E/2 cells) that showed a similar expression profile to that of human primary hepatocytes, but this strategy did not improve production of infectious particles.

Recently, a hybrid artificial liver support system was developed using animal hepatocytes cultured in a three-dimensional hollow fiber (3D/HF) system. This bioartificial liver showed several characteristic features of liver tissue for more than 4 months.[10-12] By growing our HuS-E/2 cells in a similar 3D culture[5] the gene expression profile was improved to more closely match that of human primary hepatocytes. Because the 3D cell culture condition more closely reproduces the *in vivo* environment of hepatocytes,[13] culturing these cells in this manner may support the entire HCV life cycle.

In this study we utilized this small 3D culture system and showed it to be ideal for culturing HuS-E/2 cells for the study of bbHCV infection. Using this system we are now able to study the variable patterns of the life cycle of different bbHCV strains as well as HCV-related cellular events.

Materials and Methods

Cell Culture. HuS-E/2 cells were cultured as previously described.[5] For the 3D/HF system, HuS-E/2 suspension was injected into the lumen of HF (Toyobo, Osaka, Japan) made from cellulose acetate and containing pores for nutrients and waste exchange (Supporting Fig. 1). The bundles were centrifuged to induce organoid formation. The cells in the fibers were cultured in 12-well plates (two capillary bundles per well) with gentle rotation using serum-free medium (Toyobo) in a CO_2 incubator at 37°C. The number of cells was adjusted to 3 × 105 cells per two-capillary bundle at the start of each experiment.

RNA Experiments. Total RNA was extracted from two-dimensional (2D) cultured cells, patient sera, or from 100 times concentrated culture medium as previously described.[4,5] For cells cultured in the 3D/HF, sterile scissors were used to cut each fiber into small pieces (1 mm^2 each), which were then solubilized in Sepasol RNA-1 (Nacalai Tesque, Kyoto, Japan). RNA was then extracted according to the manufacturer's protocol. Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed as described.[5]

HCV Infection. HCV infection experiments were carried out using sera from HCV patients. The amount of each inoculum was adjusted so as to add similar amount of HCV-RNA to the medium of the cells. After 24 hours, the cells were washed three times with phosphate-buffered saline (PBS) and cultured for the designated times. To assess the passage of infectivity, 12 mL of culture medium from the primary infected cells was collected, concentrated 100 times by filtration through Amicon Ultra-15, Ultracel-10K filters (Millipore, Carrigtwohill, Cork, Ireland), and 40 μL concentrated medium/well was used to infect naïve HuS-E/2 cells. All experiments were done with approval of the Ethical Committee of Kyoto University. Informed consent from patients was required for this approval.

Cloning and Sequencing. To amplify the complementary DNA (cDNA) fragment corresponding to hypervariable region 1 (HVR-1),[14] a nested RT-PCR was performed using Superscript III (Invitrogen, Carlsbad, CA) and PrimeSTAR HS DNA Polymerase (Takara, Tokyo, Japan). Reaction conditions were adjusted according to the manufacturer's protocol. Primers used were previously described[15] and are shown in Supporting Table 1. PCR products were then purified and cloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen). Ten recombinant clones were randomly isolated for each PCR product and sequenced as described.[16]

Quantitative Detection of HCV Core and Interferon alpha (IFN-α) Protein by Enzyme-Linked Immunosorbent Assay (ELISA). The culture medium of infected cells was collected and concentrated 100 times as previously mentioned for the detection of HCV-core, or used directly for detection of IFN-α. HCV core protein was quantified using the Trak-C Core ELISA (Ortho Clinical Diagnostics, Neckargemünd, Germany). IFN-α was quantified using the Human IFN-A ELISA kit (PBL Biomedical Laboratories, Piscataway, NJ). Light absorbance was then measured using a Wallac 1420 multilabel counter (PerkinElmer Life Science, Waltham, MA).

Cytotoxicity Assay. Culture medium was collected from HCV-infected cells and used for measuring lactate dehydrogenase (LDH) levels using an LDH cytotoxicity

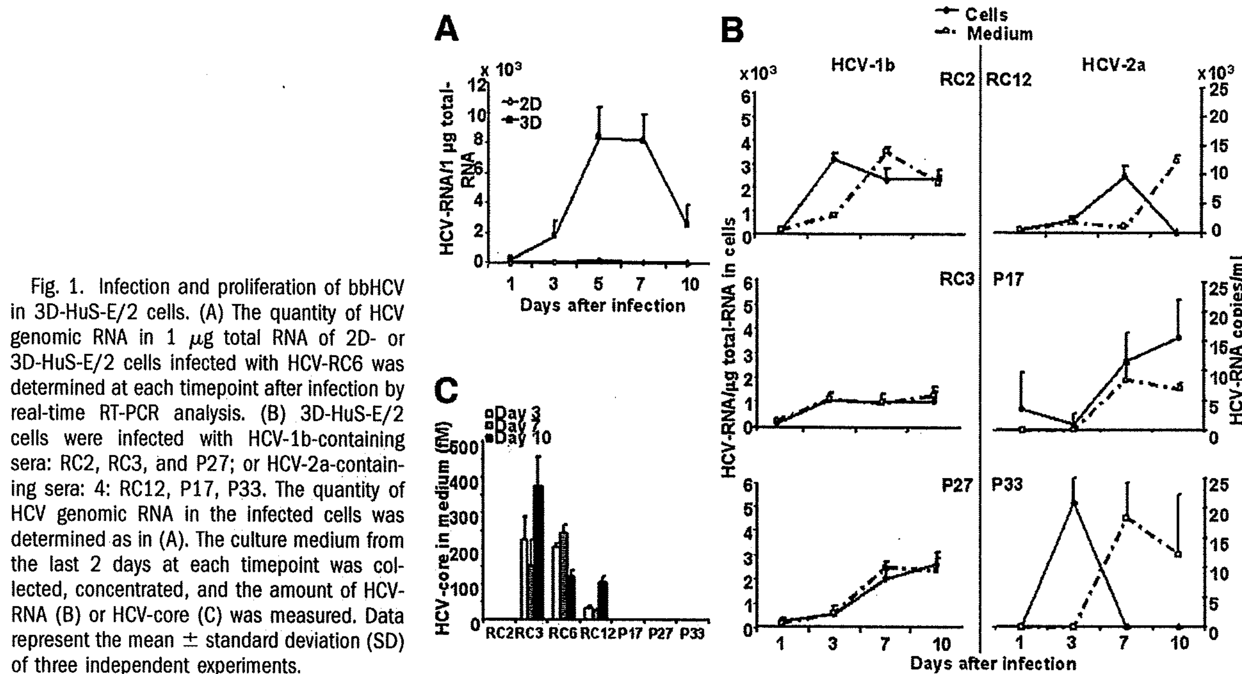

Fig. 1. Infection and proliferation of bbHCV in 3D-HuS-E/2 cells. (A) The quantity of HCV genomic RNA in 1 μg total RNA of 2D- or 3D-HuS-E/2 cells infected with HCV-RC6 was determined at each timepoint after infection by real-time RT-PCR analysis. (B) 3D-HuS-E/2 cells were infected with HCV-1b-containing sera: RC2, RC3, and P27; or HCV-2a-containing sera: 4: RC12, P17, P33. The quantity of HCV genomic RNA in the infected cells was determined as in (A). The culture medium from the last 2 days at each timepoint was collected, concentrated, and the amount of HCV-RNA (B) or HCV-core (C) was measured. Data represent the mean ± standard deviation (SD) of three independent experiments.

detection kit (Takara Biomedicals). Light absorbance was then measured as described above.

Sucrose Density Gradient. The culture medium of the infected cells was collected, concentrated 500 times, and loaded onto a 20%-50% (wt/vol) sucrose gradient containing 50 mM PBS, 100 mM NaCl, and 1 mM EDTA, followed by centrifugation at 100,000*g* for 16 hours at 4°C in a SW41Ti rotor (Beckman, Fullerton, CA). The gradient was fractionated into 31 fractions that were used for HCV-RNA and core detection and HCV infection into naïve cells as described above.

Electron Microscopy. The 1.12 g/mL fraction obtained by the sucrose density gradient showed the secondary infection activity as analyzed by transmission electron microscopy. The fraction was ultracentrifuged and the almost all supernatant was removed. The residual 10 μL of the solution was directly applied to a formvar-carbon grid for negative staining with 1% uranyl acetate solution and observed with an electron microscope (JEOL1010, JEOL, Tokyo, Japan).

Results

HuS-E/2 Cells Cultured in 3D/HF System Are Highly Permissive for Infection and Proliferation of bbHCV. We compared the ability of HuS-E/2 cells cultured in the 3D/HF system (3D-HuS-E/2 cells) to those cultured as a monolayer (2D-HuS/E2 cells) to reproduce infection by HCV genotype 1b (HCV-RC6), derived from patient serum (RC6). The HCV-RC6 RNA levels in the 3D-HuS/E2 cells were significantly higher at all timepoints (Fig. 1A), showing that the 3D/HF system greatly improves the proliferation of bbHCV in HuS-E/2 cells. We observed that both the early stages of infection and the continuous replication of HCV-RC6 in HuS-E/2 cells was improved by 3D/HF culture when the culture conditions were changed after the infection from 3D/HF to 2D and vice versa (Supporting Fig. 2).

As reported,[17] blocking CD81, an HCV-supposed entry receptor, during infection significantly impaired HCV proliferation into 3D-HuS-E/2 cells (Supporting Fig. 3), suggesting that CD81 is essential for HCV infectivity in 3D-HuS-E/2 cells. Although the expression level of CD81 mRNA in 3D-HuS-E/2 cells was observed, no significant change from 2D-HuS/E2 cells was found (data not shown), indicating that the quantity of CD81, at least, is not responsible for the improvement.

We then examined whether this system can be used for proliferation of six different bbHCV samples, three of which are HCV-1b (HCV-RC2, HCV-RC3, and HCV-P27) and three HCV-2a genotypes (HCV-RC12, HCV-P17, and HCV-P33) (Fig. 1B). Proliferation of HCV-RNA in the cells was seen in all six cases, suggesting that this system can be widely used for analysis of infection and proliferation of bbHCV strains. HCV-RNA and HCV-core were also detected in the culture medium (Fig. 1B). Different HCV strains showed variable patterns of proliferation and HCV-core secretion into the medium. Although HCV-core was detected from day 3 onward when

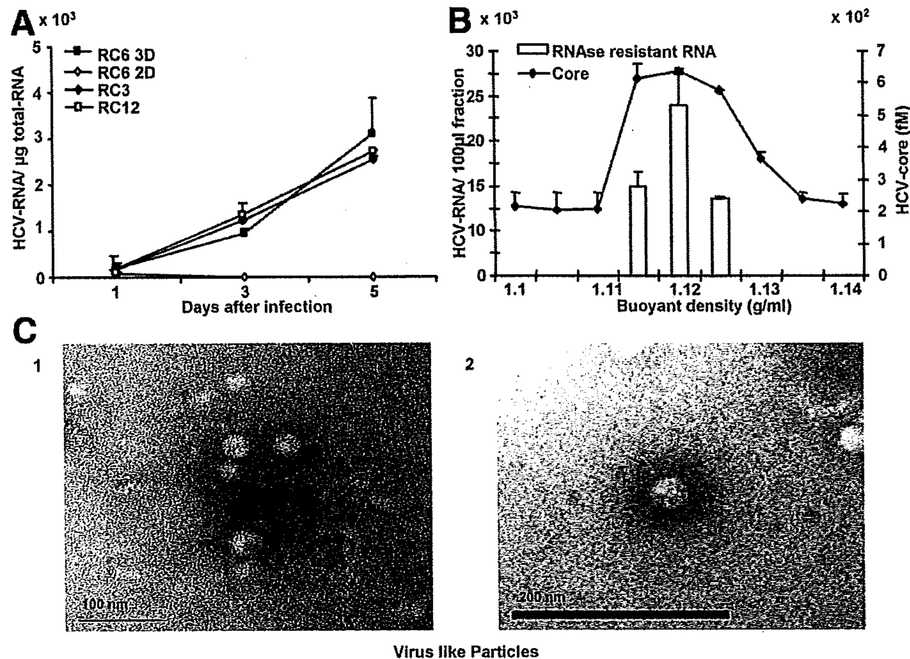

Fig. 2. Production of infectious virus-like particles from 3D-HuS-E/2 cells infected with different HCV strains. (A) The culture medium of 3D-HuS-E/2 cells infected with HCV-RC3 or HCV-RC6 was collected from days 5 to 7 p.i. and for HCV-RC12 from days 23 to 25 p.i. The culture medium of 2D-HuS-E/2 cells infected with HCV-RC6 was also collected from days 5 to 7 p.i., and used to treat naïve 3D-HuS-E/2 cells. The quantity of HCV genomic RNA in 1 μg of total cellular RNA was determined as in Fig. 1. (B) The concentrated culture medium of 3D-HuS-E/2 cells infected with HCV-RC3 was collected from days 5 to 7 p.i., and fractionated by ultracentrifugation with a 20%-50% sucrose density gradient. HCV-core protein and the RNase A-resistant HCV-RNA in the different fractions were quantitatively analyzed using an HCV-core ELISA kit and real-time RT-PCR, respectively. Data represent the mean ± SD of three independent experiments. (C) Photomicrograph showing negatively stained virus-like particles from the culture medium of HCV-RC3-infected 3D-HuS-E/2 cells (arrowheads, panels 1 and 2). The arrows indicate the spike-like structures found on the surface of the virus-like particles (panel 2).

RC3, RC6, and RC12 were used for infection, it was undetectable when RC2, P17, P27, and P33 sera were used, similar to 2D-HuS-E/2 cells infected with HCV-RC6 (Fig. 1C).

Production of Infectious Particles from 3D-HuS-E/2 Cells Infected with bbHCV. The culture media from 2D or 3D-HuS/E2 cells infected with RC6 serum (Fig. 1A) were collected from days 5 to 7 postinfection (p.i.), concentrated, and inoculated into naïve 3D-HuS-E/2 cell culture media. HCV-RNA's proliferation in the infected cells was only detected when using the culture medium from 3D-HuS-E/2 cells and not 2D-HuS-E/2 cells (Fig. 2A). Media collected from HCV-RC3 at days 5 to 7 and from HCV-RC12 from days 23 to 25 p.i. were also able to infect naïve cells (Fig. 2A). These data suggested the production and secretion of infectious virus-like particles. To investigate this further, biophysical analysis was performed. The culture medium of HCV-RC3 infected 3D-HuS-E/2 cells at day 7 p.i. was fractionated using a sucrose density gradient after RNase A treatment. HCV core was detected in the 1.11 to 1.14 g/mL fractions; similarly, the nuclease-resistant HCV RNA peaked in the 1.12 g/mL fraction (Fig. 2B). Furthermore, only the 1.12 g/mL fraction was able to infect naïve cells as examined above (data not shown). This fraction was pelleted by ultracentrifugation and examined by electron microscopy with negative staining. We observed 33-nm to 45-nm diameter spherical particles (Fig. 2C, panel 1) with spike-like structures from 7-9 nm in length on the surface (Fig. 2C, panel 2), consistent with HCV morphology reported previously in HCV patients.[18] These were detected in the sample collected from HCV-RC3-treated but not mock-treated 3D-HuS-E/2 cells. These data suggest that production of infectious virus-like particles occurs in 3D-HuS-E/2 cells infected with some bbHCV strains. It is therefore likely that 3D-HuS-E/2 cells can be used to reproduce nearly all steps in the HCV life cycle.

Prolonged Culture of HCV-Infected Cells in the 3D Hollow Fiber System. For HCV-RC6-infected cells (Fig. 3A), the amount of HCV-RNA in the cells fluctuated during the 30-day culture period. The levels of both HCV-RNA and HCV-core in the medium showed a similar pattern of fluctuations that peaked on days 5 and 20 p.i. Unlike RC6, the pattern of HCV-RNA levels in the medium of RC12-infected cells showed a negative

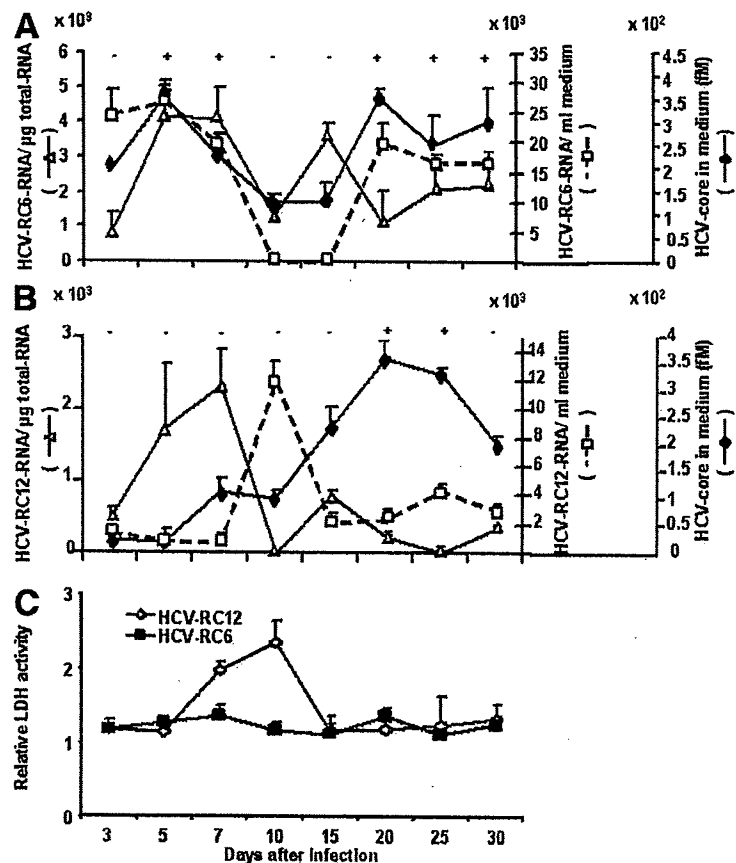

Fig. 3. Prolonged culture of HCV-infected cells in the 3D/HF system. After infection with HCV-RC6 (A) and HCV-RC12 (B), 3D-HuS-E/2 cells were cultured for 30 days with a medium change every 2 days. The HCV-RNA in the cells and medium as well as the HCV-core in the medium were quantitatively analyzed at the designated timepoints as in Fig. 1. Culture media were also used to treat naïve 3D-HuS-E/2 cells to examine the secondary infection as in Fig. 2. (+) and (−) indicate detection or no detection of secondary infection. (C) Culture media of HCV-RC6 and HCV-RC12 infected cells collected at each timepoint were used for the detection of LDH levels released from dead cells. LDH levels were normalized to uninfected cells cultured for the same time. Data represent the mean ± SD of three independent experiments.

correlation with that detected in the cells. This was clearly seen on day 10 p.i., when a sharp increase and decrease of HCV-RNA in the medium and the cells, respectively, was observed (Fig. 3B). Similarly, the amount of HCV-core detected in the medium throughout the culture was not correlated with RNA levels in the medium. Instead, core levels were very low in the first 10 days, at which time levels increased, reaching a peak on day 20 p.i. (Fig. 3B). Culture media from cells infected with HCV-RC6 from days 5 to 7 and 20 to 30 p.i. (Fig. 3A) and that from HCV-RC12 from days 20 to 25 p.i. showed passage of infectivity (Fig. 3B). All culture media showing infectivity appeared to have a high amount of HCV-core protein.

Clonal Changes in HCV During Prolonged Culture. In order to perform a populational analysis to understand the fluctuating pattern seen during HCV proliferation, two sera with limited HCV variants, HCV-RC6 (two major strains) and -RC12 (single major strain) from immunosuppressed liver transplantation patients with recurrent HCV were used in the previous prolonged infection experiment. The variants' composition was analyzed by single-strand confirmation polymorphism analysis for HCV-HVR1 (Supporting Fig. 4). RC6 serum (Fig. 4A) showed two different major sequences, HCV-RC6-1 and -2 strains, which constituted 60% and 40%, respectively, and shared 85% homology. In cells infected with HCV-RC6 the nucleotide sequence of HVR1 on day 5 showed 97% homology to HCV-RC6-1, and on day 20 p.i. it showed 97% homology to HCV-RC6-2. These data suggest selection of the dominant HCV strain in the cells over time. For RC12 (Fig. 4B), the nucleotide sequence on day 5 p.i. had only one nucleotide difference from that of the HCV from the original serum. The sequence from day 20 p.i. was four nucleotides different from that from the serum, and five different from the cells on day 5 p.i. These data indicated that each peak of HCV-RNA that appeared in the cells infected with RC12 serum included primarily a single HCV strain with a slightly different genomic sequence. This suggests that the periodic appearance of HCV-RNA peaks in the cells infected with a particular HCV strain is a result of selection and/or mutation of HCV strains during the prolonged culture period.

Cellular Response Induced by bbHCV Infection. At day 10 p.i., HCV-RNA levels in the culture medium rose and RNA levels in 3D-HuS-E/2 cells infected with HCV-RC12 dropped (Figs. 1B, 3B). To determine if this was caused by a cytotoxic effect of HCV infection, LDH levels were measured in the culture medium of HCV-RC6- and