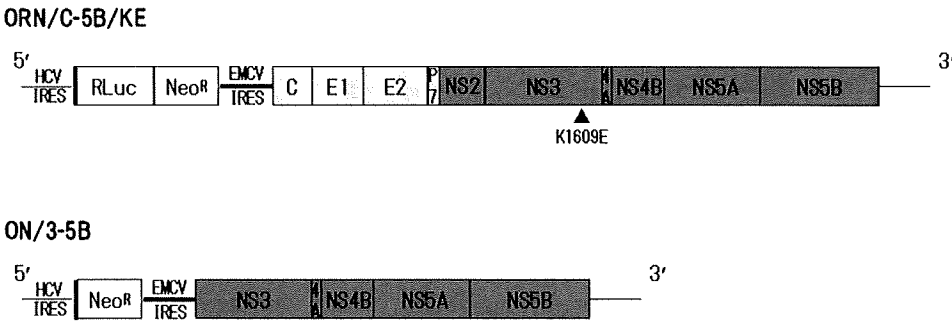

Fig. 1. Schematic gene organization of the genome-length and subgenomic HCV RNA used in this study. ORN/C-5B/KE encoding the RL gene was replicated in OR6 cells and ON/3-5B in sO cells. RL in OR6 cells was expressed as a fusion protein with neomycin phosphotransferase (Neo^R). The arrowhead indicates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

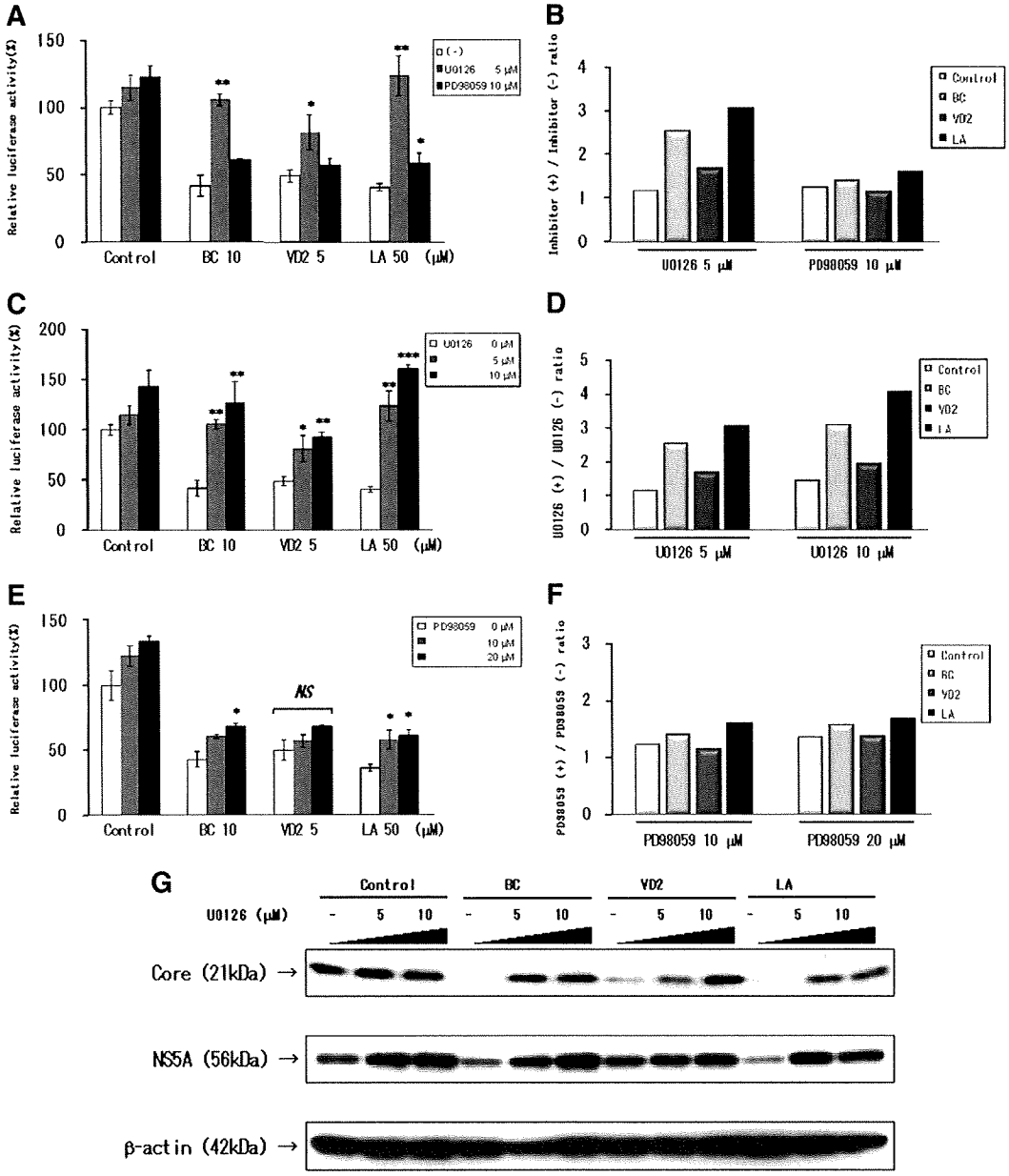

Fig. 2. U0126 strongly inhibited the anti-HCV activities of the anti-HCV nutrients BC, VD2, and LA in OR6 cells. (A,B) Effects of MEK-specific inhibitors on the three nutrients at the 60% inhibitory concentration. OR6 cells were pretreated with DMSO, 5 μM U0126, or 10 μM PD98059 for 1 hour. The cells 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 $\pm$ 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.

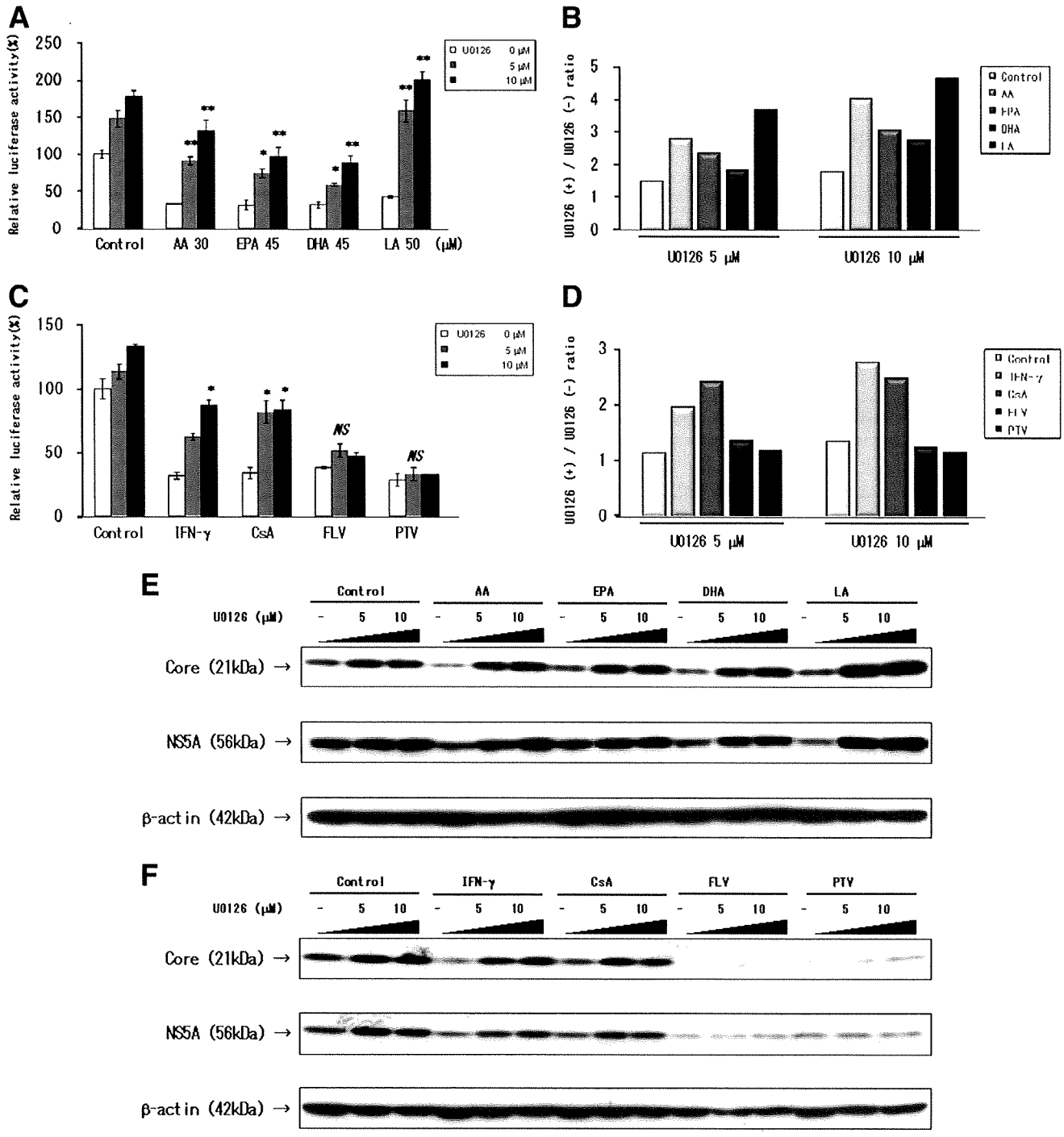

Fig. 3. U0126 dose-dependently attenuated the anti-HCV activities of PUFAs, IFN-γ, and CsA, but not the statins. (A-D) OR6 reporter assays of the dose effects of U0126 on the PUFAs and anti-HCV reagents at the 60% inhibitory concentration. OR6 cells were pretreated with DMSO or U0126 as in Fig. 2C and 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).

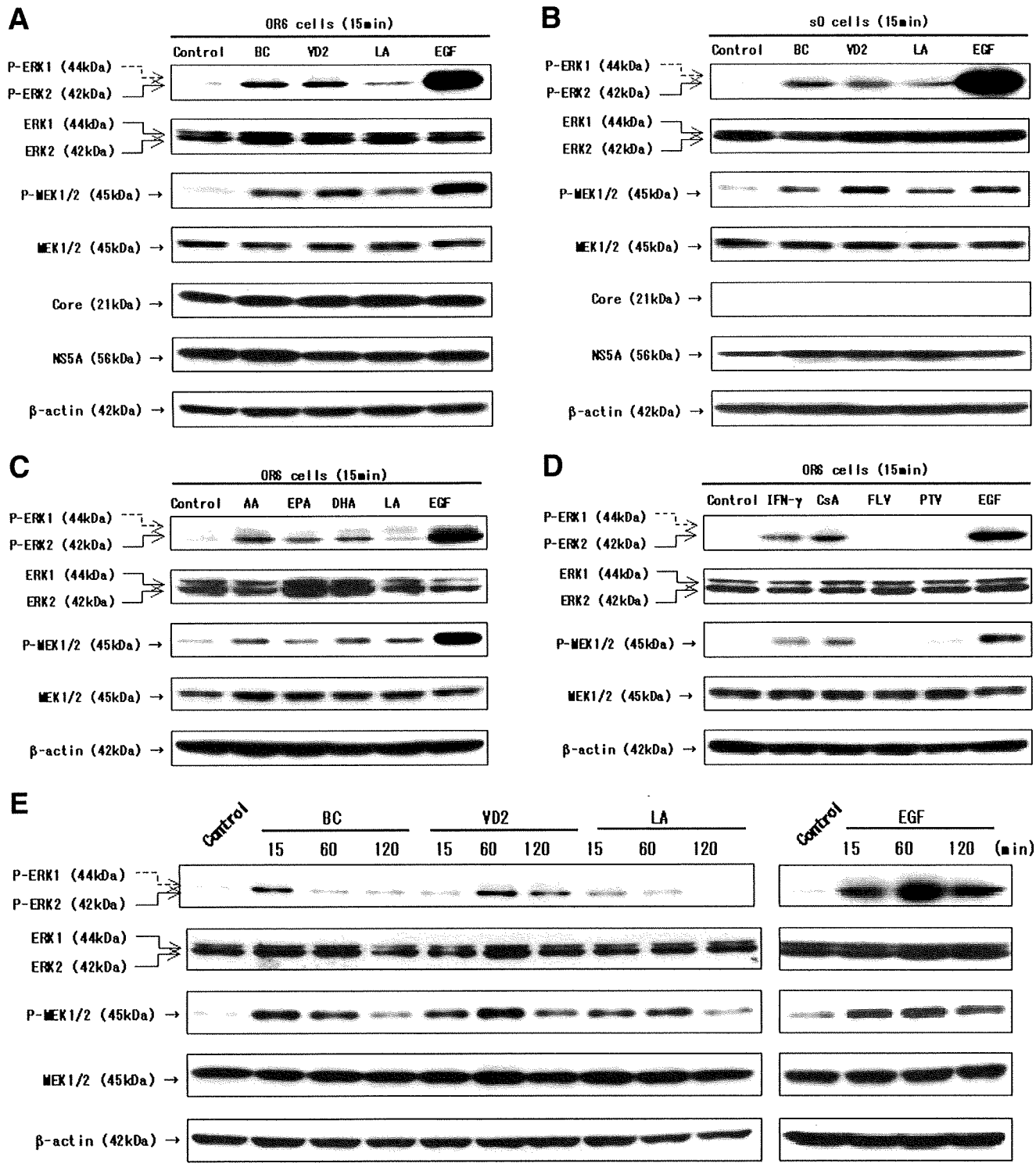

Fig. 4. U0126 attenuated the MEK-ERK1/2 signaling pathway activated by anti-HCV nutrients and reagents. (A, B) Three anti-HCV nutrients—BC, VD2, and LA—increased the phosphorylation of MEK-ERK1/2 in both full-length and subgenomic HCV RNA replication cells. OR6 cells (A) or sO cells (B) were maintained 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.

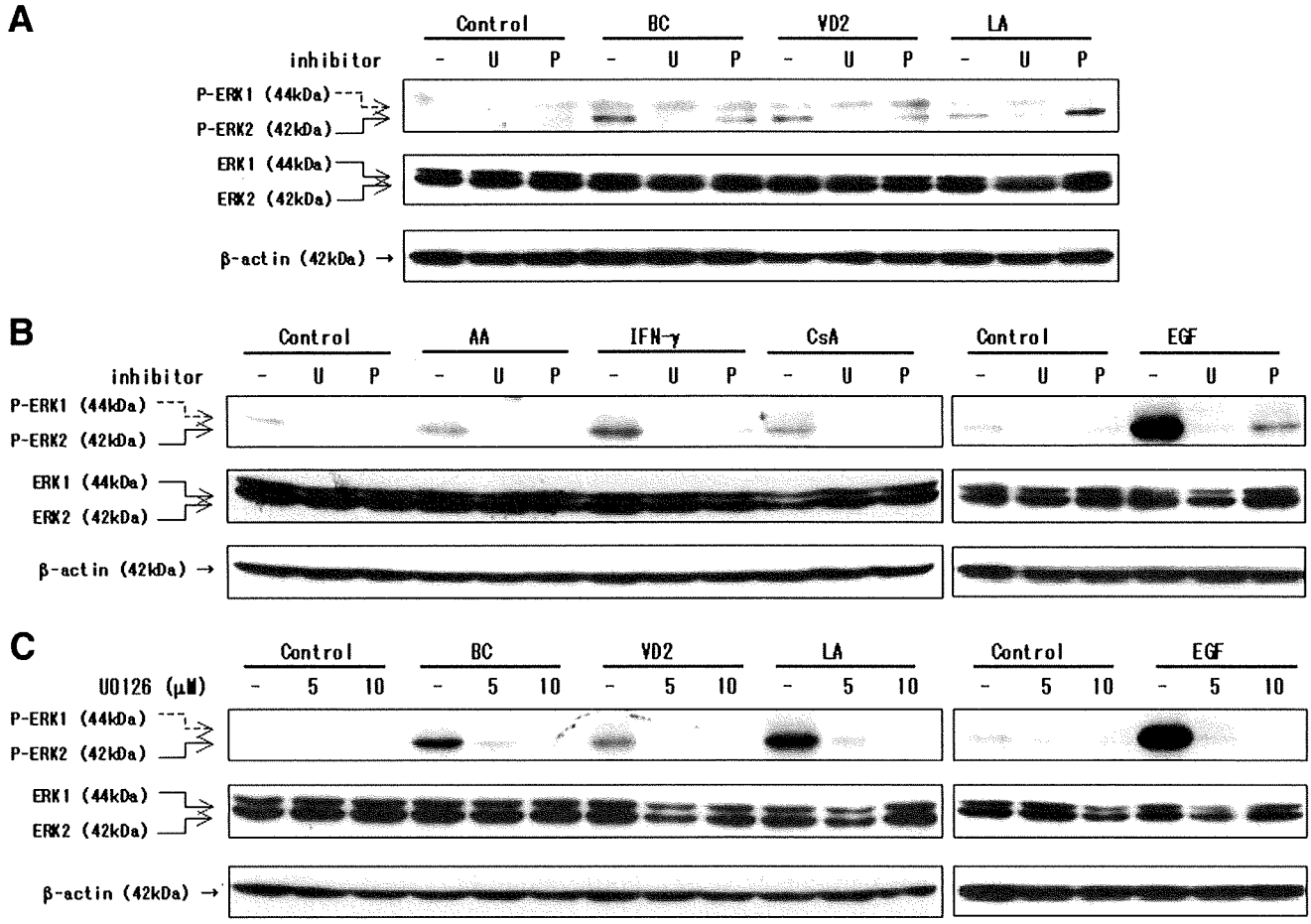

Fig. 5. U0126 strongly abolished ERK1/2 phosphorylation by the anti-HCV nutrients, anti-HCV reagents, and EGF. (A,B) Effects of the MEK1/2-specific inhibitors on ERK1/2 phosphorylation by anti-HCV nutrients and reagents. OR6 cells were precultured as described in Figs. 4A and B, and then pretreated with 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

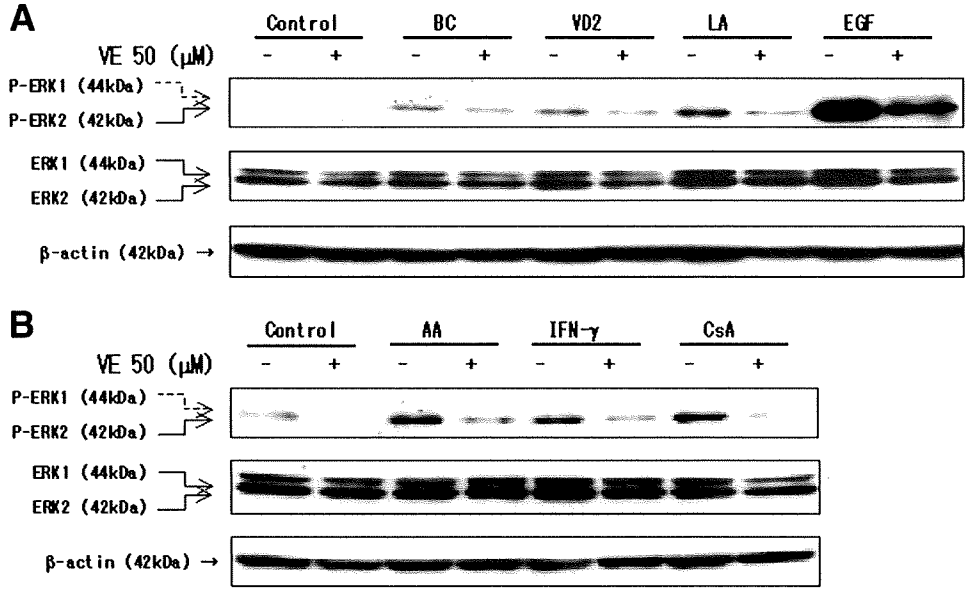

Fig. 6. VE attenuated ERK1/2 phosphorylation by the anti-HCV nutrients and reagents. OR6 cells were precultured as described in Figs. 4A and B, and then pretreated with ethanol (−) or 50 μM VE (+) for 1 hour. The cells were then treated with control medium, 20 μM BC, 10 μM VD2, 100 μM LA, or 50 ng/mL 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.

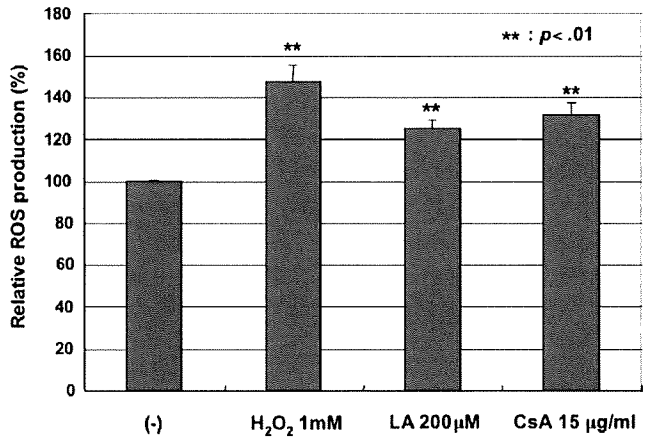

Fig. 7. ROS production by H_2O_2, LA, and CsA. OR6 cells were untreated or treated with H_2O_2 (1 mM), LA (200 μM), and CsA (15 μg/mL) and then incubated with dihydrodichlorocarboxyfluorescein diacetate. Fluorescence was measured with a fluorescence plate reader. $^{**}P < 0.01$ versus untreated cells.

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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Oncostatin M synergistically inhibits HCV RNA replication in combination with interferon-α

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ABSTRACT

Oncostatin M (OSM), a member of the interleukin-6 family, possesses various functions, including hepatocyte differentiation and suppression of melanoma cell growth. Here, we report anti-hepatitis C virus (HCV) activity of OSM as a new function of this cytokine. OSM possessed marked anti-HCV activity (50% effective concentration: 0.71 ng/ml) in an HCV RNA replication cell culture system. The most striking finding is that OSM exhibited synergistic inhibitory activity on interferon (IFN)-α even at a low concentration with weak anti-HCV activity, such as 25 pg/ml. OSM is a candidate anti-HCV reagent and may improve the current IFN therapy for patients with chronic hepatitis C.

1. Introduction

Currently the combination therapy of pegylated-interferon-α (PEG-IFN-α) with ribavirin (RBV) is available for patients with chronic hepatitis C (CH C). However, the sustained virological response (SVR) rate is still approximately 55% [1]. There is thus an urgent need for novel partners for IFN.

Oncostatin M (OSM) belongs to the interleukin (IL)-6 family, which also includes IL-6, IL-11, IL-27, ciliary neurotrophic factor, cardiotrophin-like cytokine, cardiotrophin-1, neuropoietin and leukemia-inhibitory factor (LIF) [2,3]. OSM was first reported as a cytokine produced from U-937 lymphoma cells, when it was found to inhibit the growth of melanoma cells [4]. The IL-6 family members share glycoprotein 130 (gp130) for signal transduction, and the OSM receptor consists of gp130 and its unique OSMR [5]. Recently it was reported that the IL-31 receptor also contains OSMR and forms a heterodimer with IL31RA [6]. OSMR and gp130 are highly expressed in liver, and OSM plays a significant role in the differentiation and regeneration of liver [7,8]. Therefore, OSM was used as a reagent for the differentiation of hepatocytes in vitro.

Here, we have found that OSM's anti-hepatitis C virus (HCV) activity is a new function of this cytokine. OSM synergistically inhibited HCV RNA replication in combination with IFN-α even at a low concentration with weak anti-HCV activity (20% inhibition). OSM may improve the current PEG-IFN-α and RBV therapy for patients with CH C and provide a clue toward understanding the diverse sensitivity to IFN therapy.

2. Materials and methods

2.1. Compounds and antibodies

IFN-α was purchased from Sigma (St. Louis, MO). OSM and IL-31 were purchased from R&D Systems (Minneapolis, MN). IL-6 was purchased from Acris Antibodies (Herford, Germany). LIF was purchased from Chemicon International (Temecula, CA). Anti-HCV core antibody (CP11) was purchased from the Institute of Immunology (Tokyo, Japan), and anti-HCV non-structural 5A (NS5A) antibody was the generous gift of Dr. A. Takamizawa (Research Foundation for Microbial Diseases, Osaka University). Anti-β-actin antibody was purchased from Sigma. Anti-signal transducer and activator of transcription (STAT) 1 and anti-STAT3 antibodies were purchased from BD Bioscience (San Jose, CA). Anti-phospho-STAT1 (Y701) and anti-phospho-STAT3 (Y705) were purchased from Cell Signaling Technology (Danvers, MA).

Abbreviations: SVR, sustained virological response; CH C, chronic hepatitis C; EC_{50}, 50% effective concentration; EMCV, encephalomyocarditis virus; gp130, glycoprotein 130; HCV, hepatitis C virus; PEG-IFN, pegylated-interferon; IL, interleukin; IRES, internal ribosomal entry site; LIF, leukemia-inhibitory factor; NS, non-structural; OSM, oncostatin M; RBV, ribavirin; RL, *Renilla* luciferase; RT-PCR, reverse transcription-polymerase chain reaction; STAT, signal transducer and activator of transcription

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2.2. Cell culture

The OR6 cell line is cloned from ORN/C-5B/KE (strain O of genotype 1b) RNA replicating HuH-7 cells, as described previously [9]. OR6c cells are cured OR6 cells from which HCV RNA was eliminated by IFN-α treatment, as previously described [10]. HCV-O/RLGE (strain O) is the authentic HCV RNA containing adaptive mutations of Q1112R, P1115L, E1202G, and K1609E in the NS3 region and replicates efficiently in OR6c cells [11]. Li23 and PH5CH cells were cultured as previously described [12].

2.3. OR6 reporter assay

For the *Renilla* luciferase (RL) assay, 1.5×10^4 OR6 cells were plated onto 24-well plates in triplicate and pre-cultured for 24 h. The cells were treated with OSM and/or IFN-α for 72 h. 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.

2.4. Cell growth assay

To examine OSM's activity in OR6 cell growth, 6.0×10^4 OR6 cells were plated onto 6-well plates in triplicate and were pre-cultured for 24 h. The cells were treated with OSM for 72 h, and then the number of viable cells was counted after trypan blue dye treatment, as previously described [13].

2.5. Reverse transcription and polymerase chain reaction (RT-PCR)

RT-PCR for gp130, OSMR, LIFR, IL6R, IL31RA and glyceraldehyde-3-phosphate dehydrogenase was performed by a method described previously [14]. Briefly, using cellular total RNAs (2 μg), cDNA was synthesized using M-MLV reverse transcriptase with oligo dT primer. One-tenth of the synthesized cDNA was subjected to PCR with the specific primer pairs (Supplementary materials).

2.6. Western blot analysis

For Western blot analysis to detect the expression of core and NS5A, 4×10^4 OR6c cells harboring HCV-O/RLGE RNA were plated onto 6-well plates and cultured for 24 h, and then were treated with IFN-α and/or OSM for 72 h. To detect the STATs and phosphorylated STATs, 5×10^5 OR6 cells were plated onto 6-well plates and cultured for 24 h, and then were treated with IFN-α and/or OSM. Preparation of the cell lysates, sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting were then performed as previously described [15].

3. Results

3.1. OSM inhibited HCV RNA replication in hepatoma cell line

We have tried to develop differentiated hepatocytes from mesenchymal stem cells using OSM as the differentiation reagent to establish the cell culture system for HCV RNA replication. We tested the reagents needed for differentiation, including OSM, to rule out negative activity for HCV RNA replication. In the course of this procedure, we happened to find that OSM possessed marked anti-HCV activity by using our developed full-length HCV RNA replication reporter system (OR6 assay system) [9]. This system enabled the prompt and precise evaluation of HCV RNA replication levels (Fig. 1A). OSM exhibited marked anti-HCV activity at a low concentration (50% effective concentration (EC_{50}): 0.71 ng/ml) (Fig. 1B) without cytotoxicity (Fig. 1C). OSM's anti-HCV activity was maintained at least until 96 h after a single administration of the reagent (Fig. 1D). These results indicate that OSM possesses anti-HCV activity at a concentration that

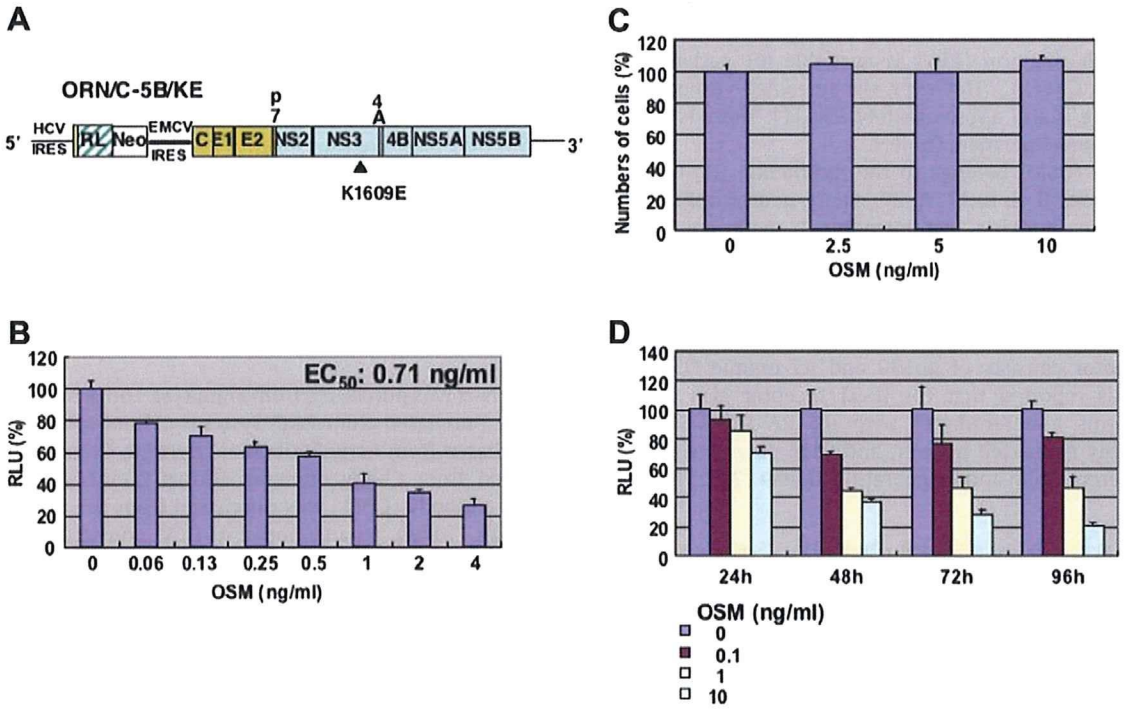

Fig. 1. Anti-HCV activity of OSM in HCV RNA replicating OR6 cells. (A) Schematic gene organization of the genome-length HCV RNA replicating in OR6 cells. The position of an adaptive mutation, K1609E, is indicated by a black triangle. (B) OR6 cells were treated with OSM for 72 h and subjected to RL assay. Relative luciferase unit (RLU) was calculated when the RL activity of the control was assigned as 100%. (C) OR6 cells were treated with OSM for 72 h and subjected to a cell viability assay with trypan blue staining. (D) OR6 cells were treated with OSM and harvested at 24, 48, 72, and 96 h and subjected to RL assay.

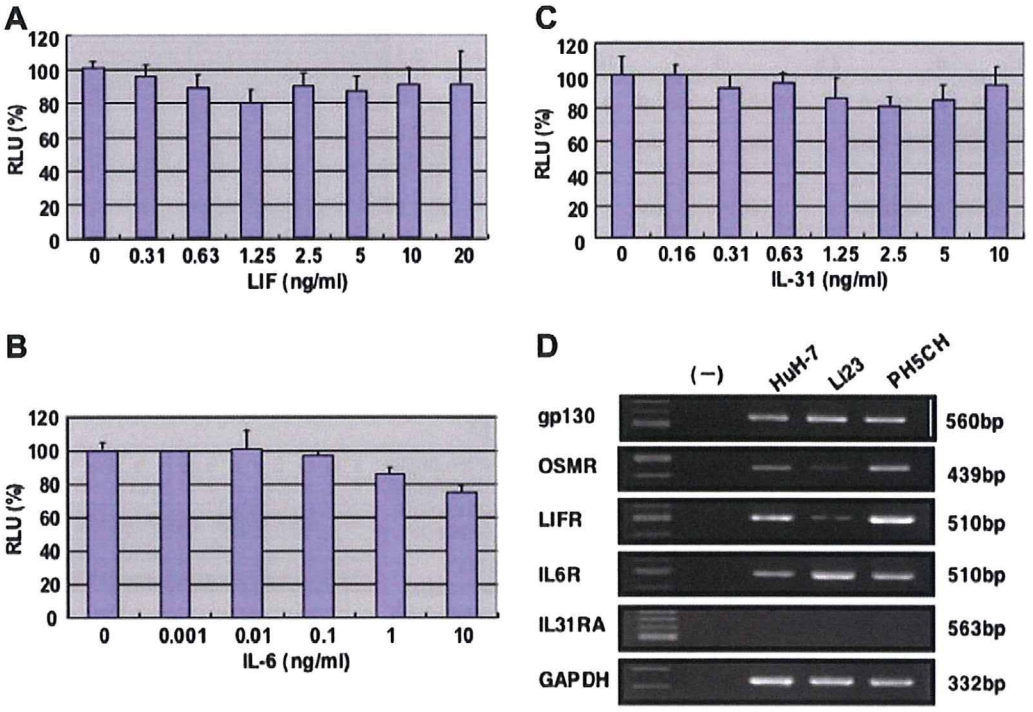

Fig. 2. The activities of LIF, IL-6 and IL-31 on HCV RNA replication. OR6 cells were treated with LIF (A), IL-6 (B) and IL-31 (C) for 72 h and subjected to RL assay. (D) RNAs from hepatocytes (HuH-7, Li23 and PH5CH) were subjected to RT-PCR with specific primer pairs to gp130, OSMR, LIFR, IL6R, IL31RA and GAPDH.

does not affect cell growth and is a new class of antiviral cytokine.

3.2. Anti-HCV activity of OSM is a unique feature in the IL-6 family

OSM belongs to the IL-6 family, whose members share the common gp130 molecule in each receptor [5]. Therefore, we next examined the activities of other representative IL-6 family members (LIF, IL-6) using the OR6 assay system. As shown in Fig. 2A, LIF had no effect on HCV RNA replication. IL-6 exhibited only a weak anti-HCV activity at the concentration of 10 ng/ml (approximately 20% inhibition) (Fig. 2B).

The OSM receptor consists of gp130 and OSMR [5]. IL31RA is another partner of OSMR and that the heterodimer of these molecules forms a receptor of IL-31 [6]. Therefore, we tried to determine whether or not IL-31 possesses anti-HCV activity in OR6 cells. The result revealed that IL-31 exhibited no anti-HCV activity. Next we examined the expression levels of the receptors in HuH-7, Li23 (a human hepatoma cell line) and PH5CH (an immortalized primary human hepatocyte line). All of these cell lines expressed gp130, OSMR, LIFR and IL6R but not IL31RA (Fig. 2D). The lack of 1L31RA expression resulted in IL-31 possessing no anti-HCV activity. These results suggest that OSM's anti-HCV activity seems to be a unique feature among IL-6 family members.

3.3. OSM synergistically enhanced anti-HCV activity of IFN-α

As HCV RNA contains three exogenous genes (RL, Neo and encephalomyocarditis virus (EMCV)-internal ribosomal entry site (IRES)) (Fig. 1A), we tried to determine whether OSM inhibits authentic HCV RNA replication in order to rule out the possibility that OSM's anti-HCV activity is not due to the inhibition of these exogenous genes. OSM inhibited core and NS5A expression in a dose-dependent manner (Fig. 3A, lanes 1–3). We next examined OSM's anti-HCV activity in combination with IFN-α using authentic HCV-O/RLGE RNA-replicating cells. OSM (1 and 10 ng/ml) drastically inhibited core and NS5A expression in combination with IFN-α (2.5, 5, and 10 IU/ml) (Fig. 3A, lanes 4–12).

OSM exhibited anti-HCV activity even at low concentrations, such as 62 pg/ml, and enhanced the anti-HCV activity of IFN-α (Fig. 3B). We also examined anti-HCV activity of CsA (0, 0.25, 0.5, and 1.0 μg/ml) alone or in combination with OSM (10 ng/ml) (Supplementary Fig. 1). OSM enhanced CsA's anti-HCV activity. Anti-HCV activity of OSM at 10 ng/ml was almost equal to that of CsA at 0.5 μg/ml. Then, we performed isobole plot analysis for EC_{50} of OSM and IFN-α. In Fig. 3C, dotted line means that the interaction of two reagents is evaluated as additive effect (or zero interaction). Points below this line correspond to synergistic interaction (or positive interaction) and points above this line indicate antagonism (or negative interaction) [16]. Therefore, isobole plot analysis of EC_{70} for OSM and IFN-α revealed that the combination of OSM and IFN-α exhibited striking synergistic inhibition of HCV RNA replication (Fig. 3C). Then we investigated whether or not OSM enhanced the IFN signaling pathway, since OSM activates STATs [17]. A kinetic study regarding the phosphorylation of STAT1 and STAT3 revealed that STAT1 (Y701) was markedly phosphorylated in the early phase within 60 min but that the phosphorylation level was reduced at 120 min (Fig. 3D). On the other hand, the phosphorylation of STAT1 by IFN-α remained consistent until 120 min after treatment (Fig. 3D). The phosphorylation kinetics of STAT3 (Y705) by OSM were consistent until 120 min (Fig. 3D). These results suggest that early-phase activation of STAT1 by OSM may trigger the synergistic activity in HCV RNA replication in combination with IFN-α.

3.4. OSM enhanced anti-HCV activity of IFN-α at even the low effective concentration by itself

As OSM exhibited marked synergistic anti-HCV activity with IFN-α, we tried to determine whether a low concentration of OSM could synergistically enhance the anti-HCV activity of IFN-

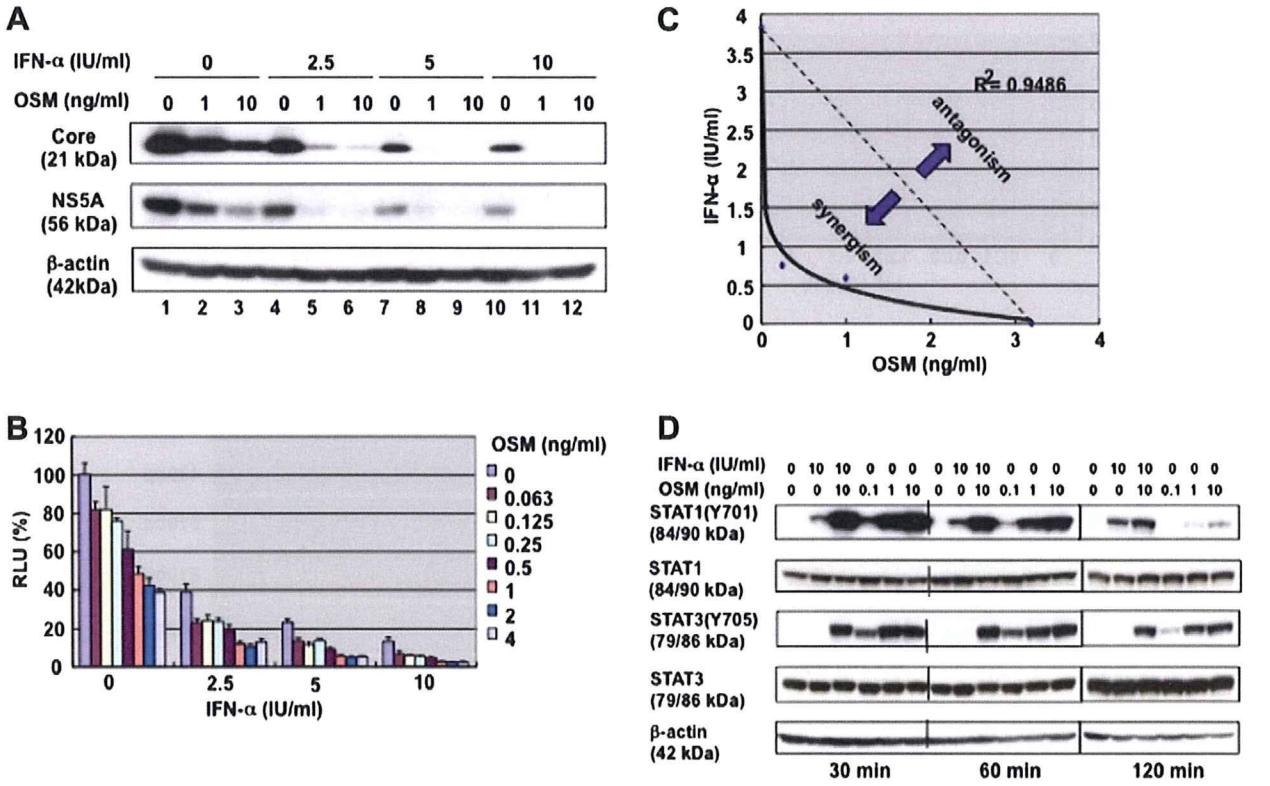

Fig. 3. Anti-HCV activity of OSM in combination with IFN-α. (A) HCV-O/RLGE-replicating OR6c cells were treated with OSM in combination with IFN-α for 96 h and subjected to Western blot analysis using anti-core, anti-NS5A and anti-β-actin antibodies. (B) OR6 cells were treated with OSM in combination with IFN-α for 72 h and subjected to RL assay. (C) Isobole plot analysis (EC_{70}) for OSM and IFN-α in OR6 cells after treatment for 72 h. (D) OR6 cells were treated with OSM and IFN-α for 30, 60 and 120 min and subjected to Western blot analysis using anti-STAT1, anti-phospho-STAT1 (Y701), anti-STAT3, anti-phospho-STAT3 (Y705) and anti-β-actin antibodies.

α. For this purpose, we treated OR6 cells with OSM at 25 pg/ml or 50 pg/ml in combination with IFN-α (0, 1, 2, 4, and 8 IU/ml). OSM alone at 25 pg/ml or 50 pg/ml exhibited only 20% inhibitory activity (Fig. 4A). However, OSM at these concentrations enhanced the anti-HCV activity of IFN-α up to 60% inhibition, when IFN-α at 8 IU/ml was treated with OSM at 25 pg/ml (Fig. 4B). These results

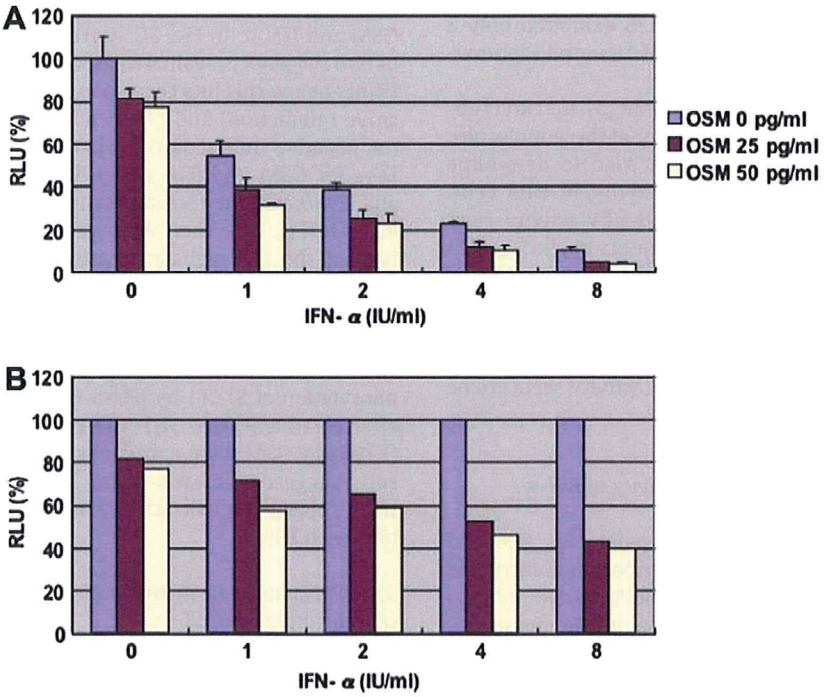

Fig. 4. OR6 cells were treated with OSM and IFN-α for 72 h and subjected to RL assay (A). Relative RL activity was adjusted when the RL activities of the cells treated with only IFN-α were assigned as 100% (B).

indicate that OSM is not only an anti-HCV reagent by itself but also a strong adjuvant for IFN-α's anti-HCV activity.

4. Discussion

In the present study, we found that OSM possesses anti-HCV activity, which constitutes a new function of this multi-functional cytokine. OSM is involved in liver regeneration and differentiation [7,8]. In the liver, OSM was produced by Kupffer cells [18], and the OSM signal was transmitted via its receptor, which consisted of gp130 and OMSR [5]. The IL-6 family members share gp130 in their receptors; it forms the heterodimer with a unique partner; for example IL6R in IL-6 and LIFR in LIF [2]. We tested the activity of LIF and IL-6 on HCV RNA replication. However, LIF did not exhibit anti-HCV activity, and IL-6 showed only weak anti-HCV activity compared to the OSM. These results suggest that OSM's anti-HCV activity is achieved via OSMR or the combination of gp130 and OSMR rather than via gp130. Recently, it was reported that IL31RA was another partner of OSMR in the IL-31 receptor [6]. If IL-31 could exhibit anti-HCV activity, OSMR seems to be significant in the signal transduction of anti-HCV activity. However, hepatocytes didn't seem to be a natural target of IL-31, because hepatocytes didn't express IL31RA. Further study is needed to clarify OSMR's role in anti-HCV activity.

Isobole plot analysis revealed that OSM exhibited a striking synergistic effect in the anti-HCV activity of IFN-α [19]. This synergistic activity of OSM may be caused by early strong activation of STAT1 by OSM. Furthermore, OSM enhanced the activity of 2′–5′ oligoadenylate synthetase promoter in combination with IFN-α (data not shown). These results suggest that STAT1 may be the key player in the synergy between OSM and IFN-α.

In this study, we found OSM's synergistic activity in the anti-HCV activity of IFN-α, when OSM was used at a low concentration (25 pg/ml) with only 20% inhibitory activity against HCV RNA replication. Surprisingly, OSM at 25 pg/ml enhanced the anti-HCV activity of IFN-α by up to 60%. RBV is the only adjuvant to the current PEG-IFN-α therapy for patients with CH C, and the combination therapy of PEG-IFN-α/RBV achieved only approximately 55% of the SVR rate. Therefore, OSM will become a strong partner to the current IFN therapy. As OSM strongly affected the anti-HCV activity of IFN-α, the serum concentration of OSM will affect the SVR in IFN therapy. The future study regarding the relationship between the serum concentration of OSM and SVR may provide a clue toward understanding the resistance to IFN therapy, and the development of OSM as a clinical reagent will serve as a breakthrough in therapy for patients with CH C.

In conclusion, we found OSM's anti-HCV activity a newly identified function of this multifunctional cytokine. The highlight of this study is that OSM exhibited a synergistic effect on the anti-HCV activity of IFN-α even at a low concentration with weak anti-HCV activity by itself.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.03.054.

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ORIGINAL ARTICLE

Double-stranded RNA-induced interferon-beta and inflammatory cytokine production modulated by hepatitis C virus serine proteases derived from patients with hepatic diseases

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Abstract We previously demonstrated that hepatitis C virus (HCV) serine protease NS3-4A was unable to cleave TRIF (adaptor protein of Toll-like receptor 3), resulting in a lack of suppression of the TRIF-mediated pathway, whereas NS3-4A cleaved Cardif (adaptor protein of retinoic acid-inducible gene I or melanoma differentiation-associated gene-5), resulting in an interruption of the Cardif-mediated pathway in non-neoplastic human hepatocyte PH5CH8 cells. To elucidate these observations, we examined the cleavage potential of NS3-4A for TRIF in PH5CH8 cells, genome-length HCV RNA-replicating O cells, and HCV-infected cells, and we demonstrated that NS3-4A lacked the ability to cleave endogenous TRIF, regardless of HCV strains derived from patients with different stages of hepatic disease. Furthermore, we demonstrated that inflammatory cytokine production by NF-κB activation via the TRIF-mediated pathway also remained unsuppressed by NS3-4A. These results suggest that the inhibitory effects of NS3-4A on antiviral signaling pathways are limited to the Cardif-mediated pathway in human hepatocytes.

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Introduction

Hepatitis C virus (HCV) infection causes a number of liver diseases such as acute hepatitis, chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [5, 24, 32, 33]. The progression of liver disease from chronic hepatitis to hepatocellular carcinoma by persistent HCV infection is a serious health problem [37]. In order to elucidate the relationship between the mechanism of persistent HCV infection and liver disease progression, it will be necessary to examine the virus life cycle and develop more effective anti-HCV reagents based on these observations. HCV is an enveloped positive single-stranded RNA (9.6 kb) virus belonging to the family *Flaviviridae* [20, 36]. The HCV genome encodes a large polyprotein precursor of approximately 3,000 amino acid (aa) residues, which is cleaved co- and post-translationally into at least ten proteins in the following order: core, envelope 1 (E1), E2, p7, non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B. These cleavages are mediated by the host and a virally encoded serine protease located in the amino-terminal domain of NS3. The serine protease activity of NS3 requires NS4A, a protein that consists of 54 aa residues, to form a stable complex with NS3 [11, 12, 19].

During infection by RNA viruses such as HCV, double-stranded RNA (dsRNA) is produced by viral RNA replication in virus-infected cells, and dsRNA is in turn recognized by Toll-like receptor (TLR) 3, which is expressed on the cell surface or in endosome vesicles [3, 13]. Additionally, dsRNA is recognized by retinoic-acid-inducible gene I (RIG-I) and/or melanoma differentiation-associated gene 5 (MDA5), which are both localized in the cytoplasm [18, 40, 41]. The stimulation of TLR3 by extracellular dsRNA leads to the activation of two signaling pathways that bifurcate at TRIF [17, 34], i.e., interferon

(IFN)-β production is induced via activation of the TRIF/TRAF3/TBK1/IRF-3 pathway, and inflammatory cytokines such as IL-6 or IL-8 are produced via activation of the TRIF/TRAF6/TAK1/NF-κB pathway (see Fig. 5a). On the other hand, the stimulation of RIG-I or MDA5 by intracellular dsRNA may induce both IFN-β and inflammatory cytokine production by similar signaling pathways that bifurcate at Cardif (i.e., the Cardif/TRAF3/TBK1/IRF-3 pathway and the Cardif/TRAF6/TAK1/NF-κB pathway) [39]. IFN-β and the inflammatory cytokines are upregulated to induce an antiviral state in virus-infected cells, and then these production levels return to a steady state in virus-eliminated cells.

Several groups, including ours, have previously reported that the HCV serine protease NS3-4A inhibited intracellular dsRNA-induced IFN-β production via the cleavage of Cardif [6, 29, 30]. The findings of these reports have indicated that Cardif is a key molecule for establishing persistent HCV infection. On the other hand, we also previously demonstrated that NS3-4A (1B-1 and O strains of genotype 1b) was not able to inhibit extracellular dsRNA-induced IFN-β production due to a lack of ability to cleave TRIF [6]; however, in another previous report, it was demonstrated that NS3-4A (N strain of genotype 1b) was able to inhibit IFN-β production via the cleavage of TRIF [27]. These latter results, taken together, suggest that among HCV strains, NS3-4A possesses a range of ability to cleave TRIF. In the present study, NS3-4As derived from patients with different stages of liver disease were used to compare the potential of NS3-4As to inhibit IFN-β production and NF-κB activation via intracellular or extracellular dsRNA.

Materials and methods

Cell culture

Non-neoplastic human hepatocyte PH5CH8 cells susceptible to HCV infection and supportive of HCV replication were cultured as reported previously [16]. Genome-length HCV RNA-replicating O cells [14], their cured Oc cells [14] and other HuH-7-derived RSc cells [25] were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carsbad, CA, USA) supplemented with 10% fetal bovine serum.

Construction of expression vectors

Retroviral vectors pCX4bsr and pCX4pur [1], which contain the resistance gene for blasticidin and puromycin, respectively, were used to construct various expression vectors. pCX4pur/myc-TRIF(P367D), (P367E), (P367D/S368C), or (P367E/S368D) mutants were constructed using PCR mutagenesis with primers containing base alterations. pCX4pur/myc-TRIF [6] was used as the template for PCR mutagenesis. The NS3-4A expression vectors used in this study were constructed using oligonucleotides (Supplementary Table S1 in Electronic Supplementary Material) as described below. RNA was extracted using an ISOGEN extraction kit (Nippon Gene, Toyama, Japan) and serum from 13 HCV-infected patients: three healthy carriers (1B-3, 1B-4, and 1B-5 strains [15]), a patient with acute hepatitis (AH1 strain [21]), and seven patients with chronic hepatitis (CH1, CH3, CH4, CH5, CH6, CH7, and CH8 strains). In addition, serum was obtained from two patients with hepatocellular carcinoma (HCC1 and HCC2 strains [2]). Informed consent was obtained from each patient before the study. The DNA fragments, including the NS3-4A region, were amplified by RT-nested PCR using KOD-plus DNA polymerase (Toyobo, Osaka, Japan) and oligonucleotides for cDNA synthesis, first-round PCR, and second-round PCR (Supplementary Table S1). The obtained DNA fragments were subcloned into the *Xba*I site of pBR322MC [22], and the nucleotide sequences of the NS3-4A regions were determined. The oligonucleotides for the construction of the NS3-4A expression vector were designed from the nucleotide sequences of the NS3-4A regions (Supplementary Table S1). The DNA fragments encoding NS3-4A were amplified by PCR using KOD-plus DNA polymerase and the specifically designed oligonucleotides, and the amplified fragments were cloned into the *Eco*RI and *Not*I sites of pCX4bsr. The nucleotide sequences of the constructed expression vectors were confirmed by Big Dye termination cycle sequencing using an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Molecular evolutionary analysis

Molecular evolutionary trees were constructed from the aa sequences of the NS3-4A regions using the UPGMA method and the program GENETYX-MAC (Software Development, Tokyo, Japan).

JFH-1 infection experiments

The infection of RSc cells with JFH-1 was performed as described previously [25]. Briefly, 1.0×10^5 RSc cells were seeded onto 6-well plates 24 h before infection. Then, an inoculum of JFH-1 was added to the cells at a multiplicity of infection of 0.1. After 96 h of JFH-1 infection, cell lysates were prepared as described below.

Immunoprecipitation and Western blot analysis

The preparation of cell lysates from PH5CH8 cells stably expressing NS3-4A and two mutants (S1165A and W1528A) [6] was performed as described previously [31].

Cell lysates were subjected to immunoprecipitation using anti-TRIF antibody (Exalpha Biologicals, Maynard, MA, USA) or anti-Cardif antibody (Bethyl Laboratories, Montgomery, TX, USA). Bound proteins were collected from cell lysates using Protein G Sepharose (Amersham) and were subjected to immunoblot analysis. Anti-NS3 antibody (polyclonal R212; a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science), and anti-NS4A antibody (C14II3-3; also a generous gift from Dr. Kohara) were used to detect NS3 and NS4A proteins. Anti-myc antibody (PL14; Medical and Biological Laboratories, Nagoya, Japan), anti-EGFP antibody (JL-8; Clontech), and anti-β-actin antibody were used in this study as primary antibodies. Immunocomplexes were detected using a Renaissance enhanced chemiluminescence assay (Perkin-Elmer Life Sciences, Boston, MA, USA).

Luciferase reporter assay

For the dual luciferase assay, we used a firefly luciferase reporter vector, pIFN-β (−125)-Luc [4], containing the IFN-β gene promoter region (−125 to +19) and pNF-κB-Luc (Stratagene). The reporter assay was carried out as described previously [8]. Briefly, a total of 0.3×10^5 cells were seeded onto 24-well plates 24 h before transfection. Then, PH5CH8 cells were transfected with 0.1 μg pIFN-β (−125)-Luc, 0.2 μg NS3-4A expression pCX4bsr vectors (NS3-4A series), and 0.2 ng pRL-CMV (Promega, Madison, WI, USA), used as an internal control reporter, for the measurement of IFN-β promoter activity. For the measurement of NF-κB promoter activity, PH5CH8 cells were transfected with 0.01 μg pNF-κB-Luc, 0.2 μg NS3-4A expression pCX4bsr vectors (NS3-4A series), and 0.02 ng pRL-CMV. The cells were cultured for 48 h, and then a dual luciferase assay was performed according to the manufacturer's protocol (Promega). In some cases, the cells were cultured for 42 h, and then poly(I-C) (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) was added to the medium for 6 h at 50 μg/ml (M-pIC) before the reporter assay. Three independent triplicate transfection experiments were conducted in order to verify the reproducibility of the results. Relative luciferase activity was normalized to the activity of *Renilla* luciferase (internal control). A manual Lumat LB 9501/16 luminometer (EG&G Berthold, Bad Wildbad, Germany) was used to detect luciferase activity.

RNA interference and real-time LightCycler PCR

siRNA duplexes targeting the coding regions of human TLR3 [31], TRIF (Dharmacon; catalog no. M-012833-00), and luciferase GL2 (Dharmacon), used as a control, were chemically synthesized. Using PH5CH8 cells with drastically decreased TLR3 or TRIF mRNA levels [6], NF-κB promoter activity was measured as described above, and dsRNA-induced inflammatory cytokine production levels were examined by using a primer set for IL-6 or IL-8 [38]. Total cellular RNA extraction and real-time LightCycler PCR were performed as described previously [6, 7].

Results

NS3-4A lacks the ability to cleave endogenous TRIF

We recently reported that NS3-4A serine protease (1B-1 strain of genotype 1b) was unable to cleave TRIF expressed in human PH5CH8 hepatocyte cells [6]. To account for this lack of cleavage ability, we examined the ability of NS3-4A to cleave TRIF mutants converted to a sequence similar to the consensus sequence required for cleavage ability by NS3-4A (Fig. 1a). The results obtained with

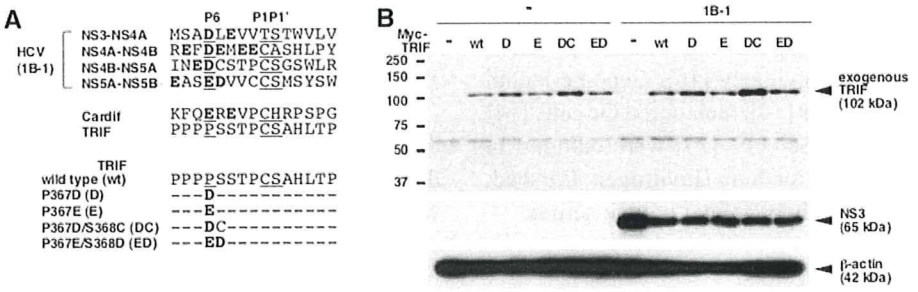

Fig. 1 NS3-4A does not cleave exogenous TRIF or its mutants at the P6 position. **a** The alignment of amino acid sequences surrounding the site cleaved *in trans* or *in cis* by NS3-4A. The consensus sequences required for cleavage by NS3-4A are underlined (P6, P1, and P1′ positions). The amino acids with a negative charge are indicated in boldface type. **b** TRIF mutants with a negative charge at the P6 position also remain uncleaved by NS3-4A. Wild-type TRIF and TRIF mutants (P367D, P367E, P367D/S368C, and P367E/S368D) are indicated as wt, D, E, DC, and ED, respectively. PH5CH8 cells stably expressing NS3-4A (1B-1) were transfected with the pCX4pur vector (as a control, –) or myc-TRIF expression vectors (wild-type strain or mutants). Production of myc-TRIF and NS3 in the cells was analyzed by immunoblot analysis using anti-myc and anti-NS3 antibody, respectively. PH5CH8 cells infected with retrovirus pCX4bsr were used as a control (–). β-actin was used as a control for the amount of protein loaded per lane

PH5CH8 cells revealed that NS3-4A (1B-1 strain) was still unable to cleave the TRIF mutants possessing D or E at the P6 position, even though an acidic aa (D or E) is known to be important for cleavage by NS3-4A [23] (Fig. 1b). Although we demonstrated that exogenously expressed Cardif, but not TRIF, was cleaved by NS3-4A (1B-1 or O strain) [6], no studies had determined whether endogenous Cardif or TRIF can be cleaved by NS3-4A. To clarify these issues, we selected anti-Cardif and anti-TRIF antibodies, and we immunoprecipitated lysates from PH5CH8 cells [in which NS3-4A (1B-1 or O strain) was overexpressed] and lysates from genome-length HCV RNA-replicating O cells [14]. We then performed immunoblot analyses using anti-Cardif, anti-TRIF, or NS3 antibodies. The results revealed that endogenous TRIF was also not cleaved by the NS3-4A expressed in PH5CH8 and O cells, whereas endogenous Cardif (75 kDa) was efficiently cleaved to the expected size (70 kDa) in PH5CH8 and O cells (Fig. 2a). On the other hand, we observed that NS3 interacted with TRIF, but not with Cardif, in both PH5CH8 and O cells (Fig. 2a), as had also been observed previously by another group [10]. In addition, we demonstrated that endogenous Cardif was cleaved by the NS3-4A/W1528A mutant, which lacks RNA helicase activity, but not by the NS3-4A/S1165A mutant, which lacks the serine protease activity (Fig. 2b). Furthermore, we examined whether or not endogenous Cardif and TRIF are cleaved in JFH-1-infected RSc cells. The results revealed that endogenous TRIF was also not cleaved in JFH-1-infected RSc cells, whereas endogenous Cardif was efficiently cleaved in these cells (Fig. 2c). In addition, we also observed that NS3 interacted weakly with TRIF, but not with Cardif, in these cells (Fig. 2c). We therefore concluded that endogenous TRIF is not cleaved by NS3-4A derived from at least the 1B-1 (genotype 1b), O (genotype 1b) or JFH-1 (genotype 2a) strain.

None of the NS3-4As derived from patients with different hepatic disease diagnoses prevented extracellular dsRNA-induced IFN-β transcription via the TRIF-mediated pathway

Although we demonstrated that NS3-4As derived from healthy carriers (1B-1 and O) was unable to suppress IFN-β production induced by the TRIF-mediated pathway [6], there is still no evidence that NS3-4As derived from patients with various hepatic disease diagnoses carry out such suppression. To obtain more evidence, we first amplified NS3-4A-encoding regions by RT-PCR using sera derived from five HCV-positive healthy carriers (including strains 1B-1 and O), one patient with acute hepatitis, seven patients with chronic hepatitis, and two patients with hepatocellular carcinoma; using these samples, we next constructed 15 types of NS3-4A expression vector. Although we observed that all of the NS3-4As expressed in PH5CH8 cells were processed into NS3 and NS4A by an intramolecular reaction, there were some size differences (60–65 kDa) of NS3 and NS4A (Fig. 3a). These size differences may be related to the aa sequence variation, as described below. Sequence analysis of these NS3-4A-encoding regions revealed that the aa sequences involved

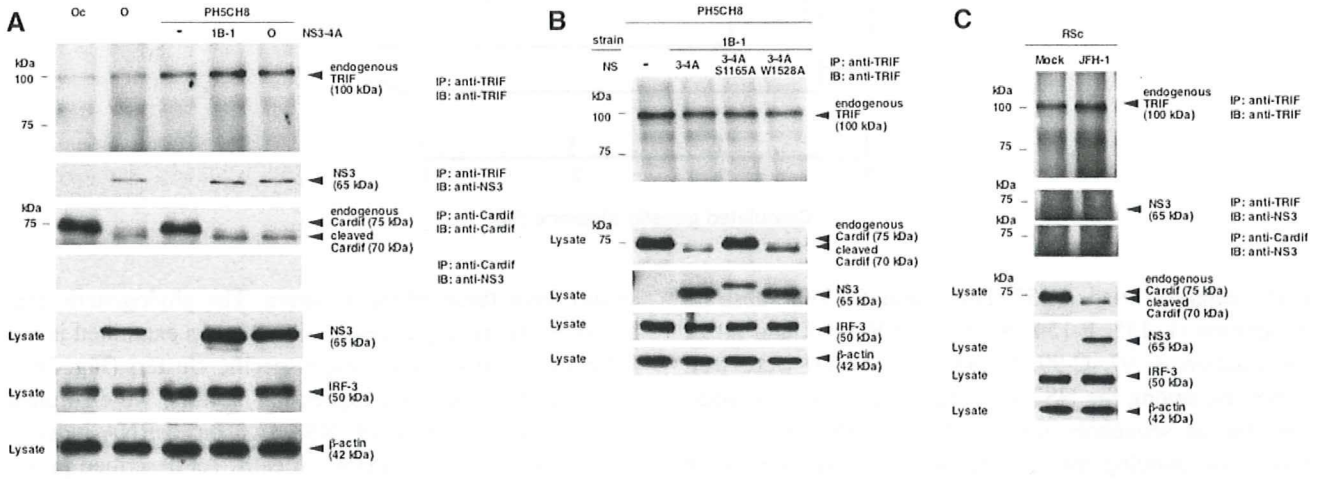

Fig. 2 NS3-4A lack the ability to cleave endogenous TRIF, but not Cardif. **a** Endogenous Cardif, but not TRIF is cleaved by NS3-4As from 1B-1 and O strains. Cell lysates were prepared and subjected to immunoprecipitation using anti-TRIF or anti-Cardif antibody. Bound proteins were collected from cell lysates using Protein G Sepharose and were subjected to immunoblot analysis using anti-TRIF, anti-Cardif, or anti-NS3 antibody. NS3, IRF3, and β-actin in the cell lysates were detected by anti-NS3, anti-IRF3, and anti-β-actin antibody, respectively. **b** Endogenous Cardif is cleaved by the serine protease activity of NS3-4A. The cell lysates were prepared and subjected to immunoprecipitation and followed by immunoblot analysis as described in **a**. **c** Endogenous Cardif, but not TRIF, is cleaved in JFH-1-infected RSc cells. The cell lysates were prepared and subjected to immunoprecipitation followed by immunoblot analysis as described in **a**

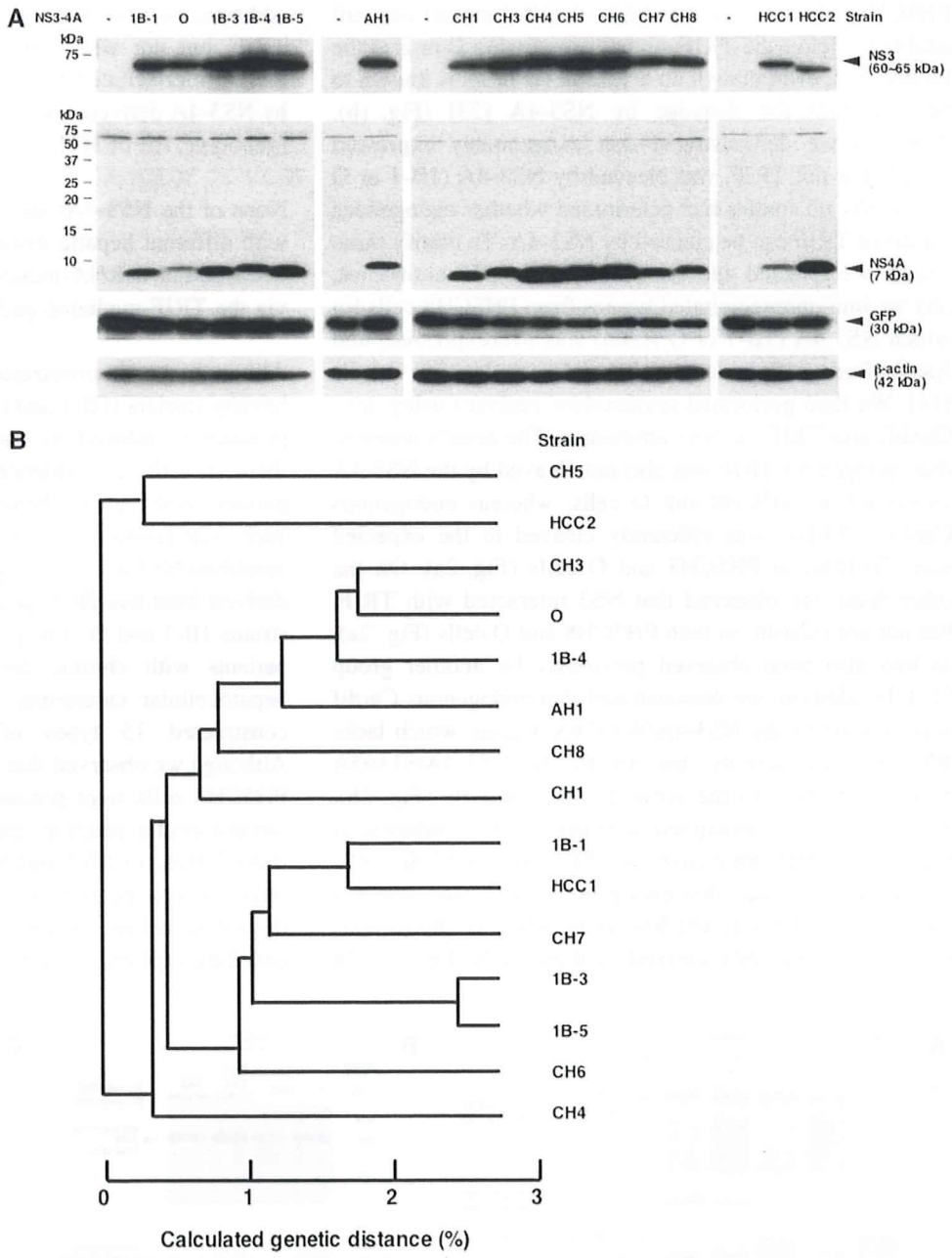

Fig. 3 Characterization of NS3-4As derived from patients with different hepatic disease diagnoses. **a** Expression of NS3 and NS4A in PH5CH8 cells. PH5CH8 cells were transfected with the expression vectors of NS3-4As derived from 15 different HCV strains and pEGFP-C1 (internal control reporter). Production of NS3 and NS4A in PH5CH8 cells was analyzed by immunoblot analysis using anti-NS3 and anti-NS4A antibody, respectively. PH5CH8 cells transfected with the pCX4bsr vector were used as a control (–). GFP was used to estimate the efficiency of transfection. β-actin was used as a control for the amount of protein loaded per lane. **b** Phylogenetic tree based on the amino acid sequences of NS3-4As used in this study

in the catalytic triad (H-57, D-81, and S-139), substrate recognition (L-135, F-154, A-157, and R-161), and metal coordination (C-97, C-99, C-145, and H-149) were well conserved among the NS3-4As (data not shown). In addition, the aa sequences (aa 626–631 in NS3, aa 1–5 in NS4A) surrounding the *cis*-cleavage site and aa 1–20 in NS4A, which is important for the stability of the NS3/4A complex, were also well conserved. The nucleotide sequences in the NS3-4 regions of these HCV strains showed differences of 6.62% (1B-4 strain)–10.47% (HCC2 strain) from those of the O strain. Similarly, the aa sequences in the NS3-4A regions of these HCV strains showed differences of 1.90% (CH3 strain)–5.11% (HCC2 strain) from those of the O strain. The phylogenetic tree based on the aa sequences of all NS3-4As examined is not indicative of any disease-stage-specific clusters (Fig. 3b).

Using these NS3-4A expression vectors, we examined the inhibitory effects of NS3-4As on dsRNA-induced IFN-β transcription in PH5CH8 cells. As described previously [6, 28], IFN-β transcription is induced via two pathways; one is mediated by the intracellular dsRNA (mainly the Cardif-mediated pathway), and the other is mediated by the extracellular dsRNA (TRIF-mediated pathway). Therefore, two different methods were used for the analysis, as described previously [6, 28]; one is to examine NS3-4A's inhibitory effects when the dsRNA