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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.)

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

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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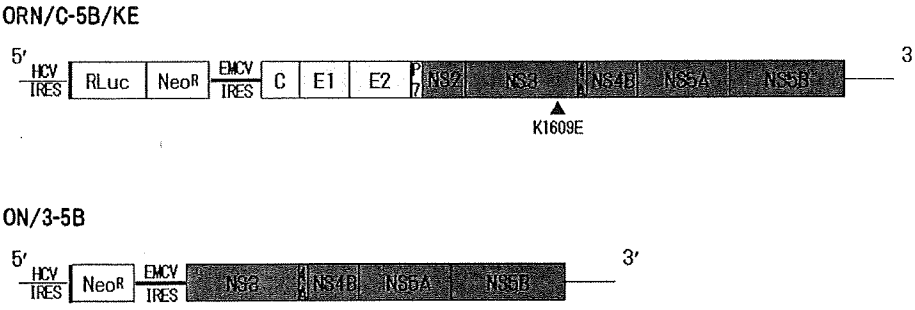

Fig. 1. Schematic gene organization of the genome-length and subgenomic HCV RNA used in this study. ORN/C-5B/KE encoding the RL gene was replicated in OR6 cells and ON/3-5B in sO cells. RL in OR6 cells was expressed as a fusion protein with neomycin phosphotransferase (Neo^R). The arrowhead indicates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

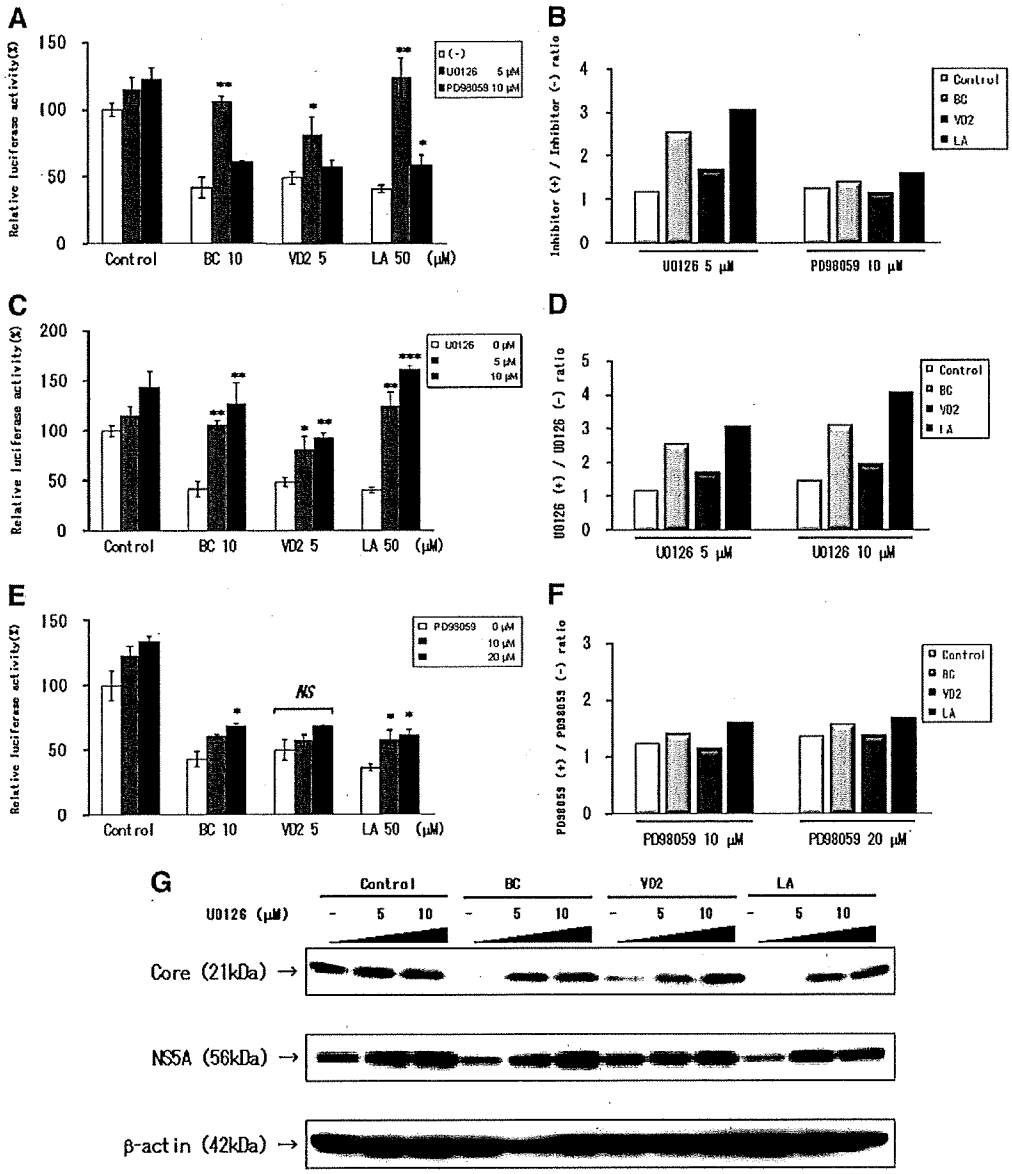

Fig. 2. U0126 strongly inhibited the anti-HCV activities of the anti-HCV nutrients BC, VD2, and LA in OR6 cells. (A,B) Effects of MEK-specific inhibitors on the three nutrients at the 60% inhibitory concentration. OR6 cells were pretreated with DMSO, 5 μM U0126, or 10 μM PD98059 for 1 hour. The cells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

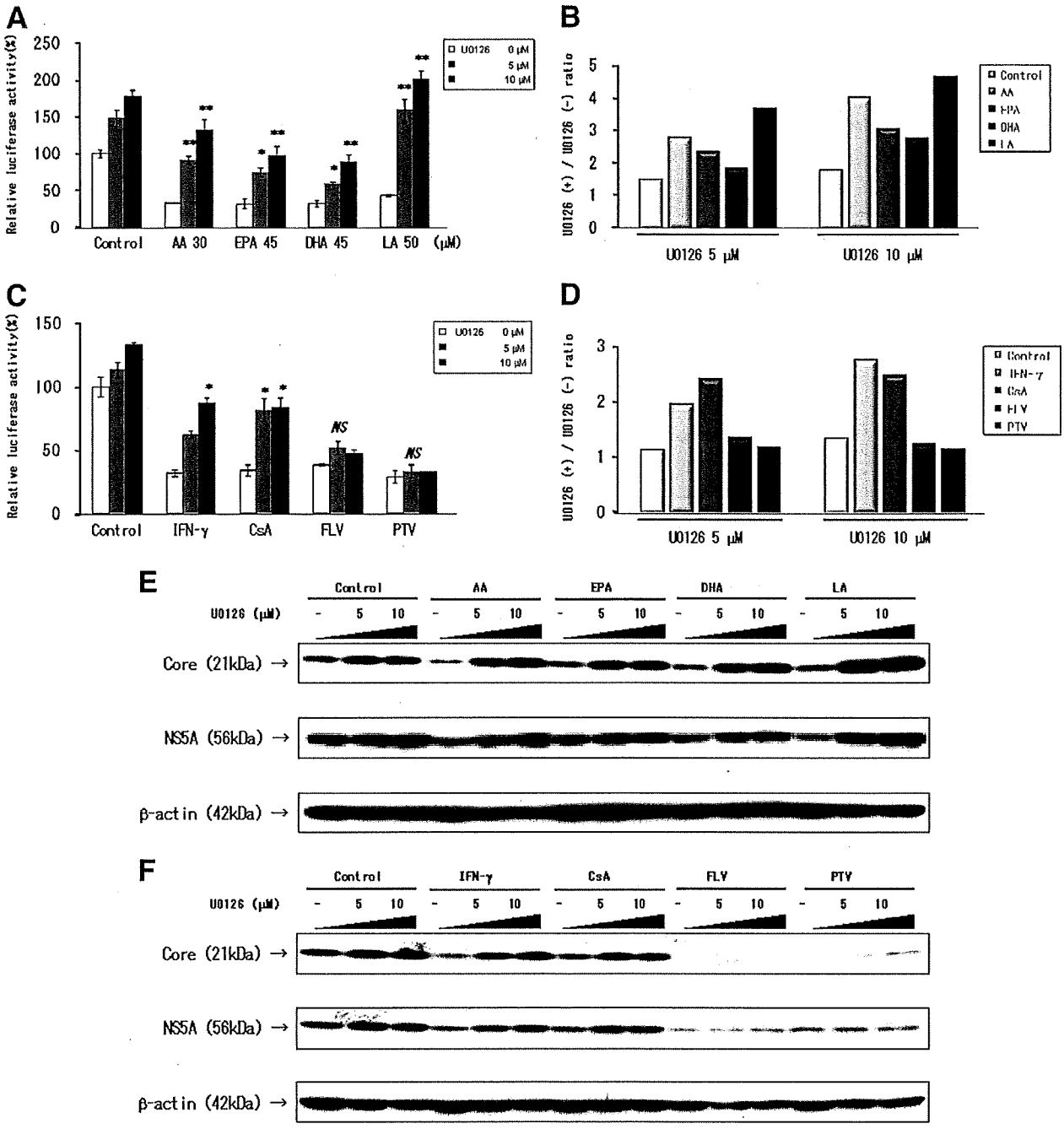

Fig. 3. U0126 dose-dependently attenuated the anti-HCV activities of PUFAs, IFN-γ, and CsA, but not the statins. (A-D) OR6 reporter assays of the dose effects of U0126 on the PUFAs and anti-HCV reagents at the 60% inhibitory concentration. OR6 cells were pretreated with DMSO or U0126 as in Fig. 2C and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

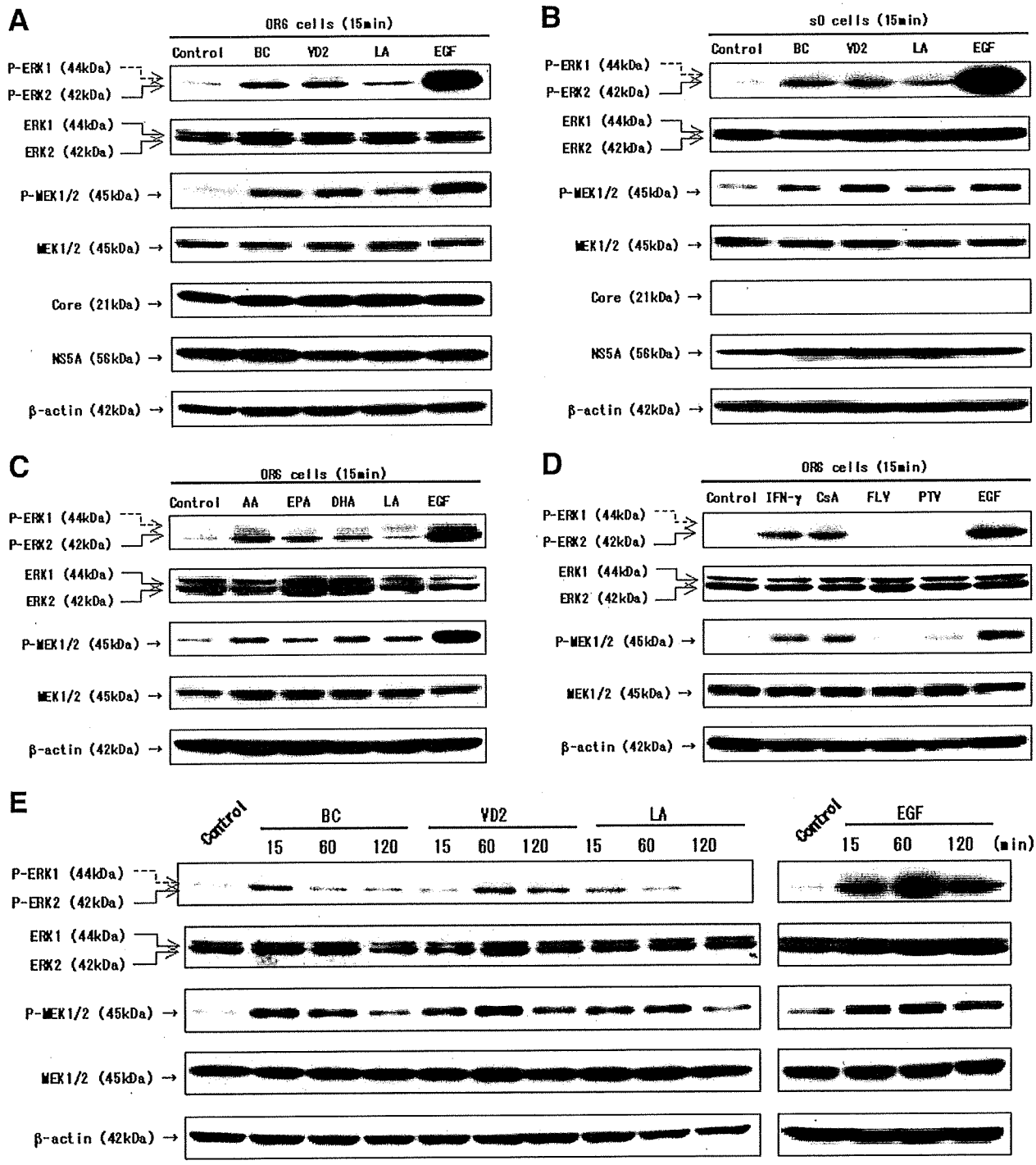

Fig. 4. U0126 attenuated the MEK-ERK1/2 signaling pathway activated by anti-HCV nutrients and reagents. (A, B) Three anti-HCV nutrients—BC, VD2, and LA—increased the phosphorylation of MEK-ERK1/2 in both full-length and subgenomic HCV RNA replication cells. OR6 cells (A) or sO cells (B) were maintained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

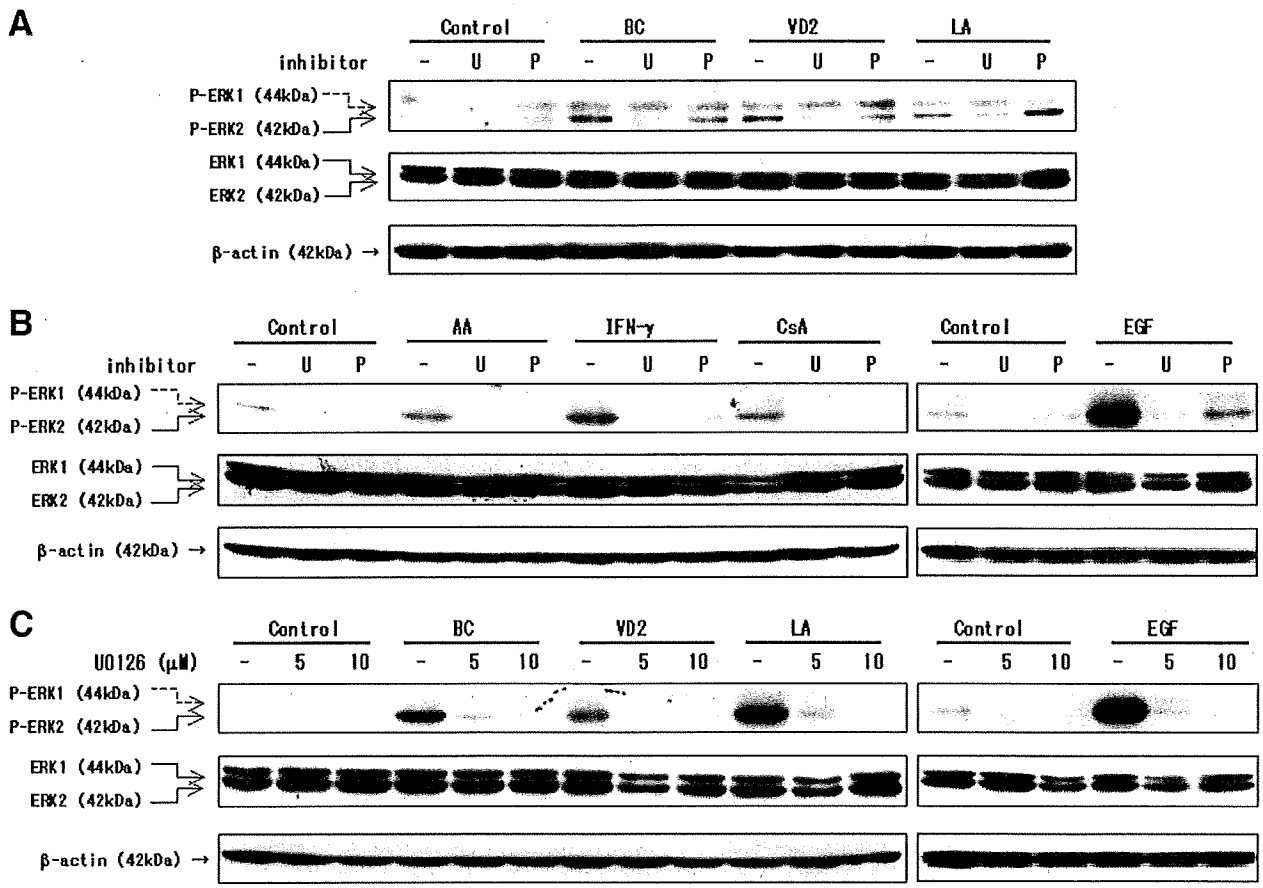

Fig. 5. U0126 strongly abolished ERK1/2 phosphorylation by the anti-HCV nutrients, anti-HCV reagents, and EGF. (A,B) Effects of the MEK1/2-specific inhibitors on ERK1/2 phosphorylation by anti-HCV nutrients and reagents. OR6 cells were precultured as described in Figs. 4A and B, and then pretreated with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

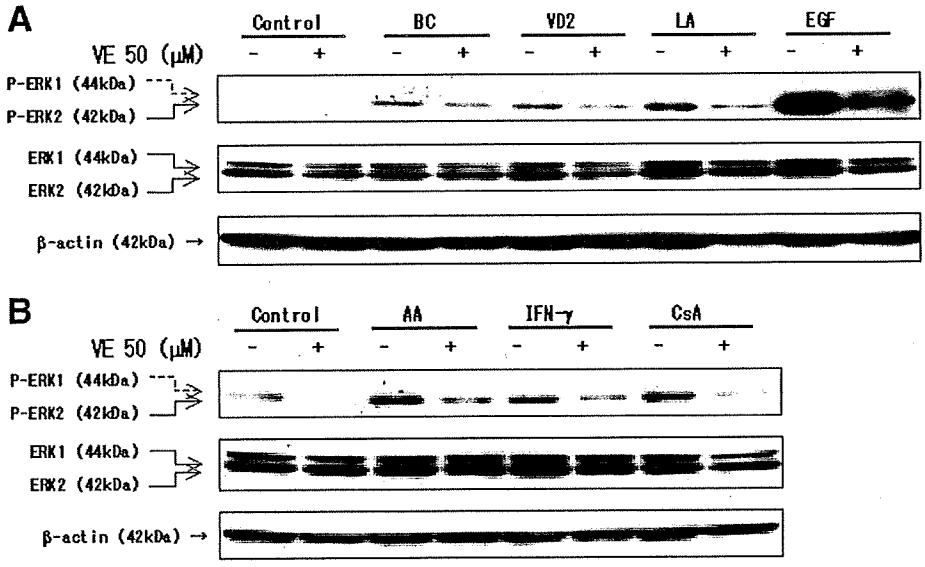

Fig. 6. VE attenuated ERK1/2 phosphorylation by the anti-HCV nutrients and reagents. OR6 cells were precultured as described in Figs. 4A and B, and then pretreated with ethanol (−) or 50 μM VE (+) for 1 hour. The cells were then treated with control medium, 20 μM BC, 10 μM VD2, 100 μM LA, or 50 ng/mL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

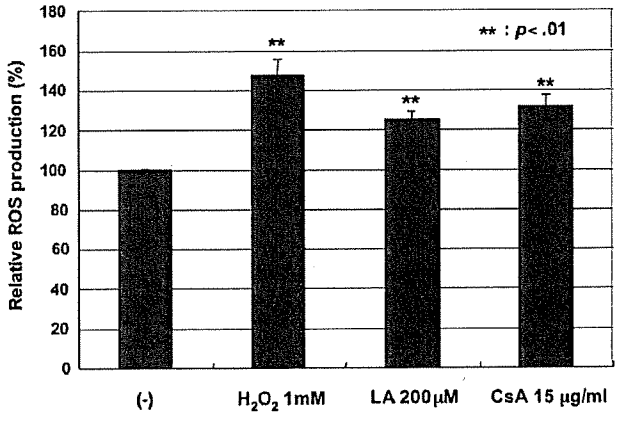

Fig. 7. ROS production by H_2O_2, LA, and CsA. OR6 cells were untreated or treated with H_2O_2 (1 mM), LA (200 μM), and CsA (15 μg/mL) and then incubated with dihydrodichlorocarboxyfluorescein diacetate. Fluorescence was measured with a fluorescence plate reader. $^{**}p < 0.01$ versus untreated cells.

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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Original Article

Clinicopathological analysis of CD133$^+$ and NCAM$^+$ human hepatic stem/progenitor cells in damaged livers and hepatocellular carcinomas

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Aim: Hepatic stem cells are capable of dramatically changing and differentiating to form mature hepatocytes in acute and chronically damaged livers; however, the clinicopathological characteristics of these heterogeneous cell populations have not been sufficiently analyzed.

Methods: In this study, cells in tissue sections from 12 cases of acute damaged livers and 31 cases of hepatocellular carcinomas (HCC), and the surrounding chronically damaged liver tissues, were analyzed by immunohistochemistry using the previously reported hepatic stem/progenitor cell marker CD133 (AC133) and the neural cell adhesion molecule (NCAM) marker.

Results: In both the acute and chronically damaged livers, CD133$^+$ cells and NCAM$^+$ cells were present in ductular reactions (DR), which include hepatic stem/progenitor cells, and became more apparent in proportion to the degree of fibrosis or histological damage. Analysis of their distribution and morphological similarities revealed that the NCAM$^+$ cell population included cells that were closer to, and morphologically more similar to, hepatocytes than were CD133$^+$ cells. Analysis of HCC using these markers revealed that 9.7% of HCC expressed NCAM (two cases had abundant NCAM$^+$ cells), while CD133$^+$ HCC were not detected.

Conclusion: These results suggest that CD133 and NCAM can be employed to enrich for hepatic stem/progenitor cells and that DR can be distinguished in greater detail using these markers. NCAM$^+$ HCC were detected, but their function remains unresolved. Expression of CD133, a potent stem cell marker, may be extremely rare in the common human HCC examined.

Key words: acute liver damage, CD133, chronic liver damage, ductular reactions, hepatocellular carcinoma, neural cell adhesion molecule

INTRODUCTION

RECENTLY, HEPATIC STEM/PROGENITOR cells possessing dual differentiation potentials and self-renewal capacities have been studied extensively as new targets for liver regeneration and treatment of hepatocellular carcinoma (HCC).[1–8] During liver damage, ductular reactions (DR) that consist of linear, circular and tubular structures, with highly variable degrees of cellular organization and heterogeneity of cell size and shape, can appear in acute and chronically damaged livers in rodents and humans.[6,9] These heterogeneous populations contain cells at various stages of differentiation, including hepatic stem/progenitor cells, but they have not been adequately analyzed clinicopathologically. Characterization of these hepatic stem/progenitor cells may help to support the study of the differentiation process and allow them to become targets for cell therapy. Recently, there have been many reports relating to the stem/progenitor cell markers CD133 and neural cell adhesion molecule (NCAM) in

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the field of stem cell research.[10,11] However, neither their presence nor their relationship within the differentiation hierarchy in chronic and acutely damaged livers, and also in HCC, have been systematically reported.

CD133 (prominin-1) was the first member of the prominin family of pentaspan membrane proteins to be identified. A cDNA cloning and tissue distribution study of AC133, which is a glycosylation-dependent epitope of CD133, revealed that AC133 is a single-chain polypeptide of 865 amino acids with a molecular weight of 120 kD. The AC133 cDNA was found to encode a five transmembrane domain molecule with an extracellular N-terminus and a cytoplasmic C-terminus, and two large extracellular loops with eight consensus sites for N-linked glycosylation.[11] While its specific function and ligands are unclear, it is well known as a cell-surface marker of primitive hematopoietic[12] and neural stem cells.[13] The similarities in topology of AC133 and mouse prominin suggested that these proteins are homologous.[14,15] Recently, mouse studies were used to show that hepatic stem cells expressed mouse prominin.[16] In rat[17] and human studies,[18] CD133 was also recognized as a hepatic stem/progenitor cell marker, and in human studies, liver cancer cell lines included CD133⁺ cells that exhibited extensive growth potential in nude mice.[19–21] Thus, CD133⁺ expressing cells are thought to be candidates for liver cancer stem cells.

Initially characterized in cells of the nervous system, the role of NCAM has been extensively analyzed. NCAM and related proteins form a large family of calcium-independent adhesion molecules that consist mainly of a varied number of immunoglobulin (Ig) domains and fibronectin type III repeats. NCAM is a cell surface sialoglycoprotein mediating homotypic and heterotypic cell–cell adhesion through a homophilic binding mechanism.[10,22] NCAM is also expressed in a variety of non-neural cell and organ types that include natural killer cells,[23] neuroendocrine glands,[24] liver,[9,25] pancreas[26] and colon,[27] and it is also expressed in a variety of cancers.[28,29] In liver, Zhou *et al.*[9] recently reported that NCAM⁺ cells exist in DR that include hepatic stem/progenitor cells in cirrhotic human livers. Immunostaining of DR with antibodies for cytokeratin 19 (CK19), NCAM and HepPar1 suggested that a differential hierarchy exists between bile duct cells and hepatocytes.

In this study, we have systematically analyzed CD133⁺ cells and NCAM⁺ cells in acute and chronically damaged livers, as well as in cancerous liver tissue, and confirmed that both CD133⁺ cells and NCAM⁺ cells exist in DR. Using serial section and double immunohistochemistry analysis, we discuss the differences between these cells with respect to their distribution and morphological similarities to hepatocytes. Finally, HCC expressing these markers were carefully analyzed to determine whether positive cells could be detected in cancerous liver tissue.

METHODS

Specimen collection

WE USED 12 previously operated cases of acutely damaged livers, which were extirpated recipient livers for transplantation, and 31 cases of chronically damaged livers, which were extirpated for resection for HCC. These resected livers were analyzed for their pathology and their redundant paraffin-embedded liver tissues were analyzed after institutional review board approval from Niigata University. The specimen profiles are described in Tables 1 and 2.

Staining

Liver tissue was fixed in 10% formalin and embedded in paraffin blocks. Sections (4 μm) were cut and mounted on silane-coated slides. Hematoxylin–eosin staining and reticulin staining were routinely performed for diagnosis. For immunohistochemistry, after removing the paraffin, antigen retrieval was performed using antigen retrieval solution (BioGenex Laboratories, San Ramon, CA, USA) for 15 min in a microwave oven. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol (Wako, Osaka, Japan) for 10 min at room temperature, and sections were incubated with primary antibodies diluted with phosphate buffered saline. The primary antibodies used were mouse anti-CK19 antibody (RCK108; Dako, Glostroup, Denmark), mouse anti-CD133 antibody (AC133; Miltenyi Biotec, Auburn, CA, USA), mouse anti-NCAM antibody (123C3; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat anti-carbomyl phosphate synthase (CPSI) antibody, a marker for mature hepatocytes (20; Santa Cruz Biotechnology). For 3,3′-diaminobenzidine (DAB) staining, slides were then stained using the Vectastain ABC Kit and DAB TRIS tablets (Muto Pure Chemicals, Tokyo, Japan). For the double peroxidase immunohistochemistry, we used DAB TRIS tablets and HISTOPRIME (Linaris-Biologische Produkte, Wertheim-Bettingen, Germany) as recommended by the manufacturer.

Tissue analysis

For the chronically damaged livers, we scored the fibrosis stages as: stage 0 (0), no scarring; stage 1 (F1),

Table 1 Clinical features of acute damaged livers

Case	Age/sex	Etiology	CD133	NCAM	AFP	ALT (max)	Time from onset to come
1	64/F	Drug?	+++	+	200	1021	> 20 days
2	42/M	Drug?	+++	++	ND	1044	> 30 days
3	51/M	Unknown	+	++	ND	1155	9 days
4	54/F	HBV	+	+	ND	13820	5 days
5	46/F	HBV	++	++	ND	6640	> 10 days
6	15/F	Wilson	+	+	ND	154	> 15 days
7	43/F	Unknown	+	+	ND	597	> 30 days
8	33/F	Unknown	+	++	ND	1177	> 30 days
9	31/F	Unknown	+++	++	3	507	60 days
10	33/M	HBV	++	++	54	1583	15 days
11	29/M	Drug?	+++	+++	23	1231	> 15 days
12	66/F	AIH	+++	+++	5	828	> 10 days

CD133 and NCAM; the frequency of positive cells in ductular reactions. +, 0–5%; ++, 6–20%; +++, ≥ 21%. AFP, α-fetoprotein (ng/mL); AIH, auto-immune hepatitis; ALT, alanine aminotransferase; HBV, hepatitis B virus; NCAM, neural cell adhesion molecule; ND, not done; Wilson, Wilson's disease.

minimal scarring; stage 2 (F2), scarring has occurred and extends outside the areas in the liver that contain blood vessels; stage 3 (F3), bridging fibrosis is spreading and connecting to other areas that contain fibrosis; and stage 4 (F4), cirrhosis. Histological activity was scored according to the METAVIR algorithm:[30] 0 (A0), none; 1 (A1), mild; 2 (A2), moderate; and 3 (A3), severe. To evaluate the frequency of $CD133^+$ or $NCAM^+$ cells we scored them as: grade 1, 1–10 positive cells per portal area and septal region; grade 2, 11–100 cells per portal area and septal region; grade 3, 101–500 cells per portal area and septal region; and grade 4, 501 or more cells per portal area and septal region. These scores were evaluated on an average of four randomly selected views.

Statistical analysis

To analyze the data for CD133 and NCAM grades according to their levels of fibrosis or histological activity scores, we performed post-test analysis with a Mann–Whitney *U*-test using SPSS software. We considered *P*-values less than 0.05 as statistically significant.

RESULTS

Clinical aspects

Acutely damaged livers

LIVER TISSUE WAS taken from four men and eight women with ages ranging from 15–66 years. One case was of autoimmune hepatitis, one case was of Wilson's disease, three cases were of hepatitis B and seven cases were of unknown etiology (Table 1).

Chronically damaged livers

Liver tissue was taken from 25 men and six women with ages ranging from 49–81 years (mean ± standard deviation [SD], 69.0 ± 8.7 years). We checked all cases for serum hepatitis virus markers. Four cases were positive for hepatitis B virus surface antigen (HBsAg), 17 cases were positive for hepatitis C virus antibody (HCV Ab), and 10 cases were negative for both HBsAg and HCV Ab. Serum α-fetoprotein (AFP) levels and serum alanine aminotransferase (ALT) levels ranged 2–10 272 μg/L and 15–126 IU/L (mean ± SD, 47.5 ± 26.5 IU/L). Fibrosis scores were: 0, one patient; 1, five patients; 2, 11 patients; 3, seven patients; and 4, seven patients. Histological activity scores were: 0, two patients; 1, six patients; 2, 17 patients; and 3, six patients. The differentiation status for HCC was graded as: well differentiated, 10 patients; moderately differentiated, 18 patients; and poorly differentiated, three patients (Table 2).

$CK19^+$ DR contain a heterogeneous population and include abundant $CD133^+$ cells and $NCAM^+$ cells

In all acute and chronically damaged livers, DR consisting of linear, circular and tubular structures with poorly defined lumens were present in a variety of degrees. To detect DR in acute and chronically damaged livers, we first stained with anti-CK19 antibody (Fig. 1). Massive necrotic and regenerative areas, with nodule formation,

Table 2 Clinical features of chronically damaged livers

Case	Age/sex	Etiology	Fibrosis stage	Activity stage	CD133	NCAM	NCAM (in HCC)	HCC	AFP	ALT	Special instructions
1	78/M	NBNC	F4	A2	2	3	+++	mod	15	49	Post-portal embolization
2	74/M	HCV	F4	A3	2	1	–	por	161	35	Post TACE
3	77/F	HCV	F3	A2	1	1	–	mod	54	75	
4	81/M	HCV	F3	A2	1	1	–	mod	9	46	
5	70/M	HCV	F2	A1	1	1	+++	mod	11	33	Post IFN therapy
6	77/M	NBNC	F1	A1	1	1	–	well	226	77	
7	49/M	HBV	F4	A2	3	3	–	por	490	53	
8	62/M	HBV	F2	A2	1	1	–	well	7	93	
9	56/M	HCV	F4	A2	2	1	–	well	2	40	
10	73/F	HCV	F2	A2	1	1	–	mod	4	19	
11	77/F	HCV	F2	A2	4	4	–	mod	7	59	Portal blood flow ↓
12	63/M	NBNC	F3	A3	2	4	–	mod	5	19	Post TACE
13	73/F	HCV	F2	A2	1	2	–	mod	1441	42	Post PEIT
14	64/M	HCV	F2	A2	1	1	–	mod	10	33	
15	62/F	HCV	F3	A2	1	1	–	well	30	126	
16	70/M	NBNC	F2	A2	1	2	–	mod	95	105	
17	75/M	HCV	F4	A2	1	2	–	mod	56	48	
18	78/M	HCV	F4	A2	1	2	–	mod	7	23	
19	71/M	HCV	F3	A3	1	1	–	mod	11	55	
20	20/F	HCV	F2	A2	1	1	–	well	56	29	
21	81/M	NBNC	F0	A0	1	1	–	well	3	15	
22	65/M	NBNC	F2	A3	3	4	+	mod	10272	33	Portal thrombosis (+)
23	60/M	HBV	F2	A2	1	1	–	mod	46	46	Post TACE
24	69/M	HCV	F3	A3	2	2	–	well	100	69	
25	55/M	NBNC	F2	A2	1	2	–	well	10	29	
26	70/M	HCV	F1	A1	1	1	–	mod	30	22	
27	81/M	NBNC	F1	A1	1	1	–	por	4488	37	Post CDDP i.a.
28	71/M	HCV	F4	A3	3	1	–	well	31	72	
29	66/M	NBNC	F3	A1	1	1	–	well	7	36	
30	71/M	NBNC	F1	A0	1	1	–	mod	230	22	Post TACE
31	51/M	HBV	F1	A1	1	1	–	mod	66	32	

AFP, α-fetoprotein; alanine aminotransferase; CDDP, cisplatin; HCC, hepatocellular carcinoma; IFN, interferon; PEIT, pericutaneous ethanol injection therapy; TACE, transarterial chemoembolization; mod, moderately differentiated; por, poorly differentiated; well, well differentiated; HBV, hepatitis B virus surface antigen (HBsAg)$^+$; HCV, hepatitis C virus surface antibody$^+$; NBNC, HBsAg$^-$ and hepatitis B virus surface antibody$^-$.

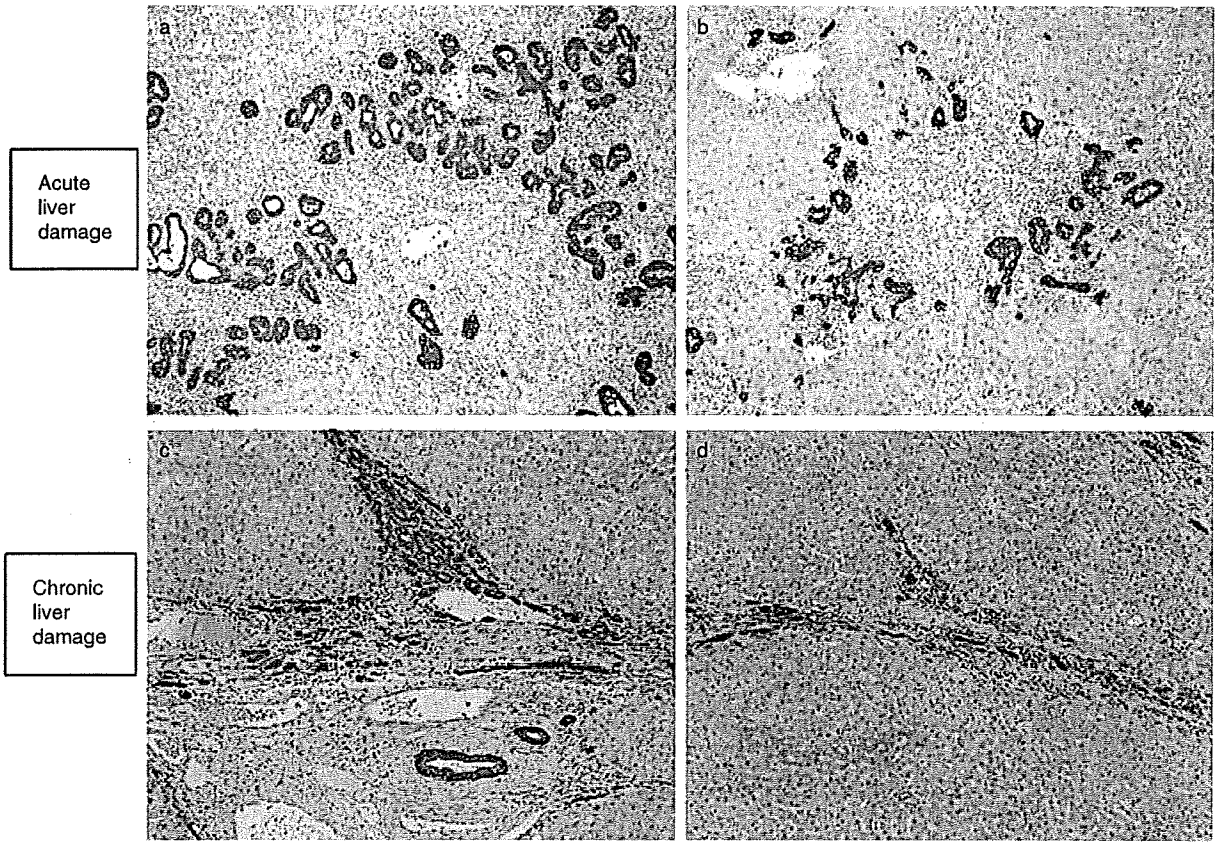

Figure 1 CK19$^+$ cells in acute and chronically damaged livers. In acutely damaged livers, a massive necrotic area (a) and regenerative area (b) can be seen. The massive necrotic area (a) contains extensive proliferation of CK19$^+$ ductular reactions (DR), while the regenerative area (b) in the same liver has fewer CK19$^+$ DR. In chronically damaged livers, CK19$^+$ DR were located mainly in the areas of portal (c) and septal (d) fibrosis. Similar results were seen in other patients. Original magnification × 100. (a,b) Case 11 in Table 1; (c,d) case 1 in Table 2.

existed in the acutely liver damage recipient livers. It was particularly noticeable that the massive necrotic area (Fig. 1a) included more DR than the regenerative area (Fig. 1b). In the chronically damaged livers, DR were present mainly in the portal mesenchyma (Fig. 1c) and in regions of septal fibrosis (Fig. 1d).

To analyze whether DR include stem/progenitor cell marker-expressing cells, liver tissues were stained for the stem/progenitor cell markers CD133 and NCAM. In all acute and chronically damaged livers, CD133$^+$ cells and NCAM$^+$ cells were found in DR (Fig. 2). Serial section analysis revealed that CD133$^+$ cells and NCAM$^+$ cells also stained positive for CK19 (data not shown). CD133 is expressed at the apical region of DR and NCAM is expressed at the cell surface of DR both in acute and chronically damaged livers (Fig. 2). CD133$^+$ cells and NCAM$^+$ cells were rarely detected in mature bile ducts with defined lumens (data not shown).

Next, we analyzed the chronically damaged liver tissues, and determined the frequencies of CD133$^+$ cells and NCAM$^+$ cells by scoring the positive cells in the portal area and septal region (Fig. 3) for their levels of fibrosis and histological activity. CD133$^+$ cells and NCAM$^+$ cells were rarely detected in cases scoring 0 or 1 for fibrosis and 0 or 1 for histological activity (Fig. 3). In particular, CD133$^+$ cells and NCAM$^+$ cells were rarely detected in case 5 in Table 2 (the patient achieved a sustained viral response to interferon) and case 21 in Table 2 (F0, A0). In most cases, there was an apparent trend in which the numbers of CD133$^+$ cells and NCAM$^+$ cells began to expand from 2 or 2 and further expanded as their fibrosis and histological damage levels

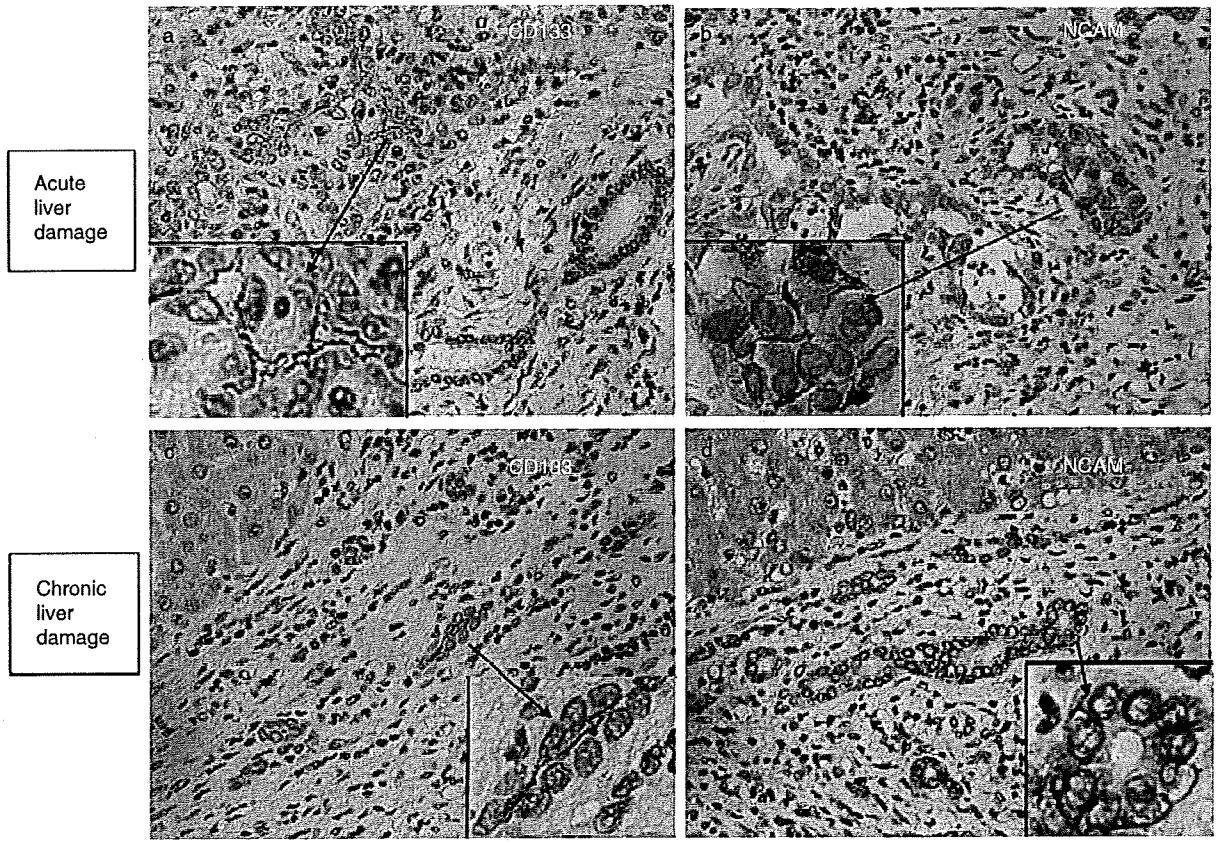

Figure 2 CD133⁺ cells and neural cell adhesion molecule (NCAM)⁺ cells in acute and chronically damaged livers. In acute (a,b) and chronically (c,d) damaged livers, CD133 (a,c) was expressed at the apical region of ductular reactions (DR), while NCAM (b,d) was expressed at the cell surface of DR. Similar results were seen in other patients. Original magnification × 200. (a,b) Case 11 in Table 1; (c,d) case 7 in Table 2.

increased. Three cases were excluded from this trend (cases 11, 12 and 22 in Table 2). In these cases, the CD133⁺ and NCAM⁺ cells were massively expanded with respect to their fibrosis and histological activity levels. It should be noted that the livers in all three cases were damaged before operation. In case 11, portal blood flow was decreased due to an unknown cause. Case 12 was treated by transarterial chemoembolization (TACE) before operation, and case 22 had portal thrombosis. In these livers, CD133⁺ cells and NCAM⁺ cells may have expanded massively due to the preoperative damage. While these three cases had abundant CD133⁺ cells and NCAM⁺ cells, there was no remarkable elevation of serum levels for AFP and ALT (Table 2). The A3 grade scores of CD133 and NCAM were significantly higher than those of the lower grade score groups (A0 + A1) for CD133 and NCAM, respectively. Furthermore, the F4 grade scores of CD133 and NCAM were significantly higher than those of the lower grade score groups (F0 + F1) of CD133 and NCAM, respectively (Fig. 3a,b).

NCAM⁺ cells include cells that are similar to hepatocytes with respect to their distribution and morphology

To study the differences between CD133⁺ cells and NCAM⁺ cells, cell size, shape and distribution were analyzed. CD133⁺ cells were relatively smaller than NCAM⁺ cells, and some NCAM⁺ cells possessed a larger nucleus and an aggregated nucleolus, as found in hepatocytes (Fig. 4, Fig. 5a,b). While serial section analysis and double immunohistochemistry analysis in acute and chronically damaged livers revealed that CD133⁺NCAM⁻ DR, CD133⁺NCAM⁺ DRs, and CD133⁻NCAM⁺ DR do exist, CD133⁺NCAM⁻ DR were

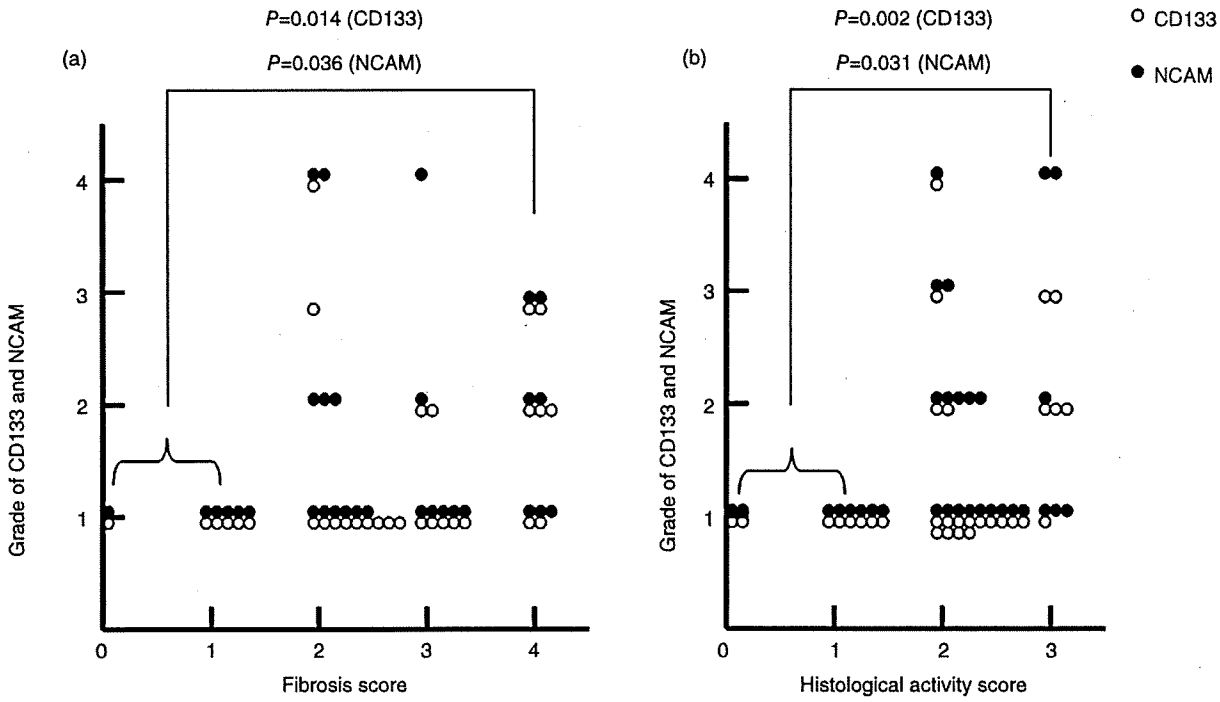

Figure 3 Grading of CD133$^+$ and neural cell adhesion molecule (NCAM)$^+$ cells according to their fibrosis and histological activity scores in chronically damaged livers (a,b). Both CD133 and NCAM were graded 1 in all cases scoring 0 or 1 for fibrosis (f) and 0 or 1 for histological activity (a). CD133$^+$ cells and NCAM$^+$ cells began to expand from F2 and A2. The A3 grade scores of CD133 and NCAM were significantly higher than those of the low grade score groups (A0 + A1) for CD133 and NCAM, respectively. Furthermore, the grade F4 scores of CD133 and NCAM were significantly higher than those of the low grade score group (F1 + F0) for CD133 and NCAM, respectively.

distributed relatively nearer to the portal vein and CD133$^-$NCAM$^+$ DR were distributed relatively nearer to hepatocytes (Fig. 4). Furthermore, while NCAM$^+$ cells were often in contact with CPSI$^+$ mature hepatocytes and had a similar morphology, we were unable to detect CPSI$^+$NCAM$^+$ mature hepatocytes (Fig. 5c,d). These results suggested that NCAM$^+$ cells include cells that are closer to the hepatocytic lineage cells than are CD133$^+$ cells.

NCAM$^+$ cells exist in certain moderately differentiated HCC while CD133 could not be detected

Finally, to explore the possibility that CD133$^+$ cells and/or NCAM$^+$ cells are cancer stem cells, or that cancer and stem/progenitor cells share common markers, 31 cases of recently operated HCC were stained with CD133 and NCAM antibodies. CD133$^+$ cells could not be detected in HCC using our methods, while CD133$^+$ DR could be detected in fibrotic tissue in, or around, the tumor (Fig. 6a,b). On the other hand, 9.7% of HCC (three cases) possessed NCAM$^+$ cells (Table 2). Surprisingly, NCAM$^+$ cells only existed in moderately differentiated HCC (16.7% of moderately differentiated HCC expressed NCAM). The HCC expressing NCAM were composed of heterogeneous cell populations. In one HCC nodule, both NCAM$^+$ cells and NCAM$^-$ cells were found to co-exist. In addition, two cases of NCAM$^+$ HCC showed a large number of NCAM$^+$ cells present in clusters (Fig. 6c,d).

DISCUSSION

RECENTLY, DR, WHICH contain hepatic stem/progenitor cells, and cholangiocytic and hepatocytic lineage cells have been intensively studied.[9,31,32] In this study, our first step involved the systematic analysis of DR using the well-known stem/progenitor markers CD133 and NCAM. Both CD133$^+$ cells and NCAM$^+$ cells can be detected in DR in acute and chronically damaged