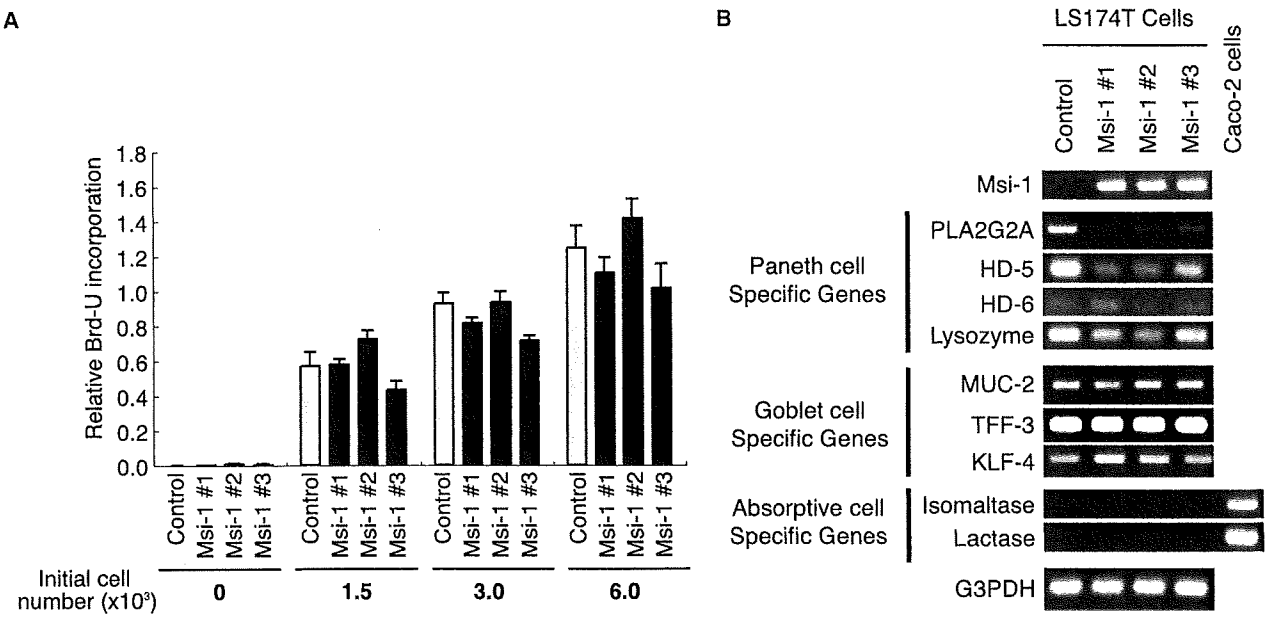

Fig. 2. Expression of Msi-1 does not change cell proliferation, but decreases expression of Paneth cell-specific genes in LS174T cells. **A** Bromodeoxyuridine (*Brd-U*) incorporation of the generated cell lines. Cells were cultured at various densities and subjected to Brd-U incorporation analysis using an enzyme-linked immunosorbent assay. Results of LS174T/GFP cells served as a control. Error bar represents SD. **B** Semiquantitative RT-PCR analysis of lineage-specific genes in the generated cell lines. Total RNA was isolated from LS174T/GFP cells (*Control*) or LS174T/Msi-1 cells (*Msi-1 #1*, *Msi-1 #2*, and *Msi-1 #3*), and subjected to analysis. A corresponding sample prepared from Caco-2 cells served as a positive control for absorptive cell-specific genes. Glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) served as an internal control

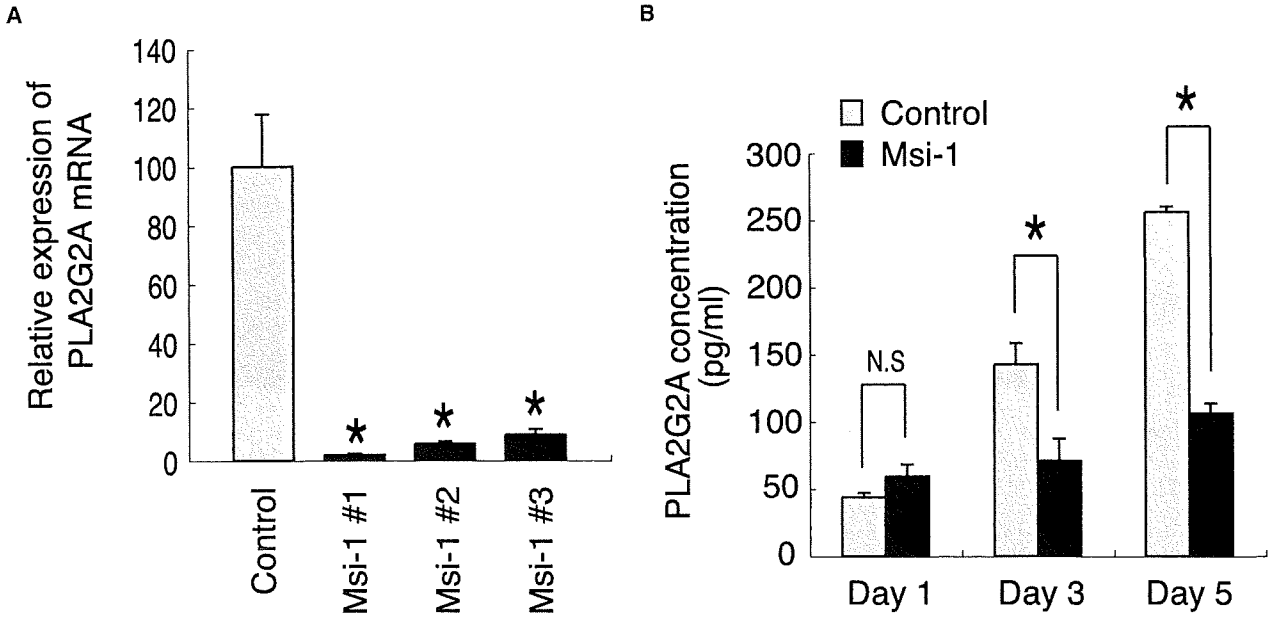

Fig. 3. Expression of Msi-1 suppresses gene expression of *PLA2G2A* in LS174T cells. **A** Quantitative RT-PCR analysis of *PLA2G2A* expression in the generated cell lines. Results of LS174T/GFP cells served as a control. Error bar represents SD. $*P < 0.05$, Student's *t* test. **B** Analysis of PLA2G2A protein secretion by LS174T parental cells (*Control*) or LS174T/Msi-1 cells (*Msi-1*). Control or LS174T/Msi-1 cells were cultured for up to 5 days, and the amount of secreted PLA2G2A was measured by enzyme immunoassay at different time points. Error bar represents SD. $*P < 0.05$, Student's *t* test

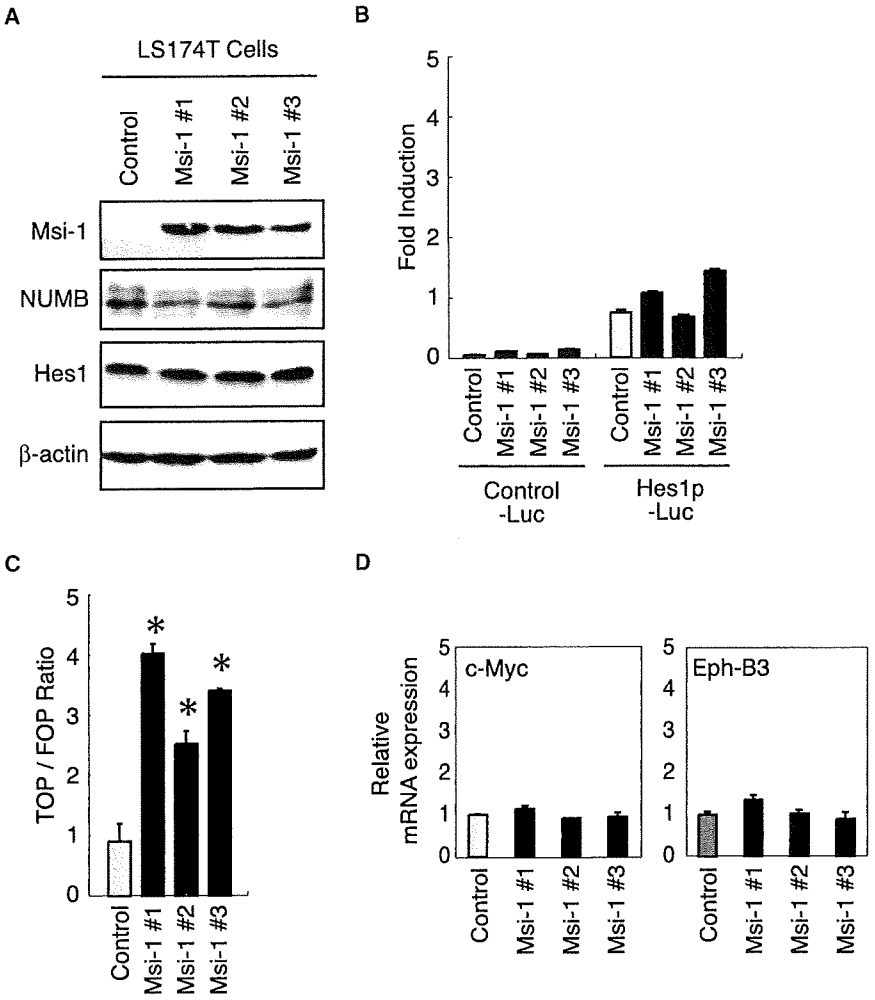

Fig. 4. Expression of Msi-1 has no effect upon expression of target genes downstream of Notch or Wnt pathways in LS174T cells. **A** Immunoblot analysis of Notch pathway genes in the generated cell lines. Total cell extract prepared from LS174T/GFP cells (*Control*) or LS174T/Msi-1 cells (*Msi-1 #1*, *Msi-1 #2*, and *Msi-1 #3*) was subjected to analysis. Result for *m-Numb* shows two distinct bands, one representing the short (*upper band*) and the other the long (*lower band*) isoform of the *m-Numb* gene. **B** Luciferase reporter assay for analysis of RBP-Jk dependent transcriptional activity. Hes1p-Luc contains six tandem repeats of RBP-Jk binding sites, whereas Control-Luc contains only the core promoter element derived from the chicken *β-actin* gene. Each data point was normalized by the corresponding Renilla luciferase activity. Error bar represents SD. **C** Luciferase reporter assay for analysis of TCF-dependent transcriptional activity. Firefly luciferase activity of the TOP-flash or FOP-flash vector was normalized by the corresponding Renilla luciferase activity. Data are shown as ratio of normalized TOP-flash and FOP-flash activity. Error bar represents SD. $^*P < 0.05$ compared with control cells, Student's *t* test. **D** Quantitative RT-PCR analysis of Wnt target genes in the generated cell lines. Relative expression level of c-Myc and Eph-B3 are shown. Error bar represents SD. Expression in LS174T/GFP cells (*Control*) is set to 1

epithelial cells,[20] we found that TCF-dependent transcriptional activity was upregulated two- to four fold in LS174T/Msi-1 cells compared with LS174T/GFP cells (Fig. 4C). However, expression of c-Myc and Eph-B3 showed no significant change between LS174T/Msi-1 cells and LS174T/GFP cells (Fig. 4D), suggesting that an additional increase of TCF-dependent transcriptional activity in these cells may not necessarily lead to changes in the expression level of the downstream target genes. These results showed that expression of Msi-1 in LS174T cells has only a minimal effect upon Notch or Wnt target genes, and suggested that changes observed in LS174T/Msi-1 cells, such as cell morphology or *PLA2G2A* expression, are presumably mediated through a mechanism independent of such molecular signaling pathways.

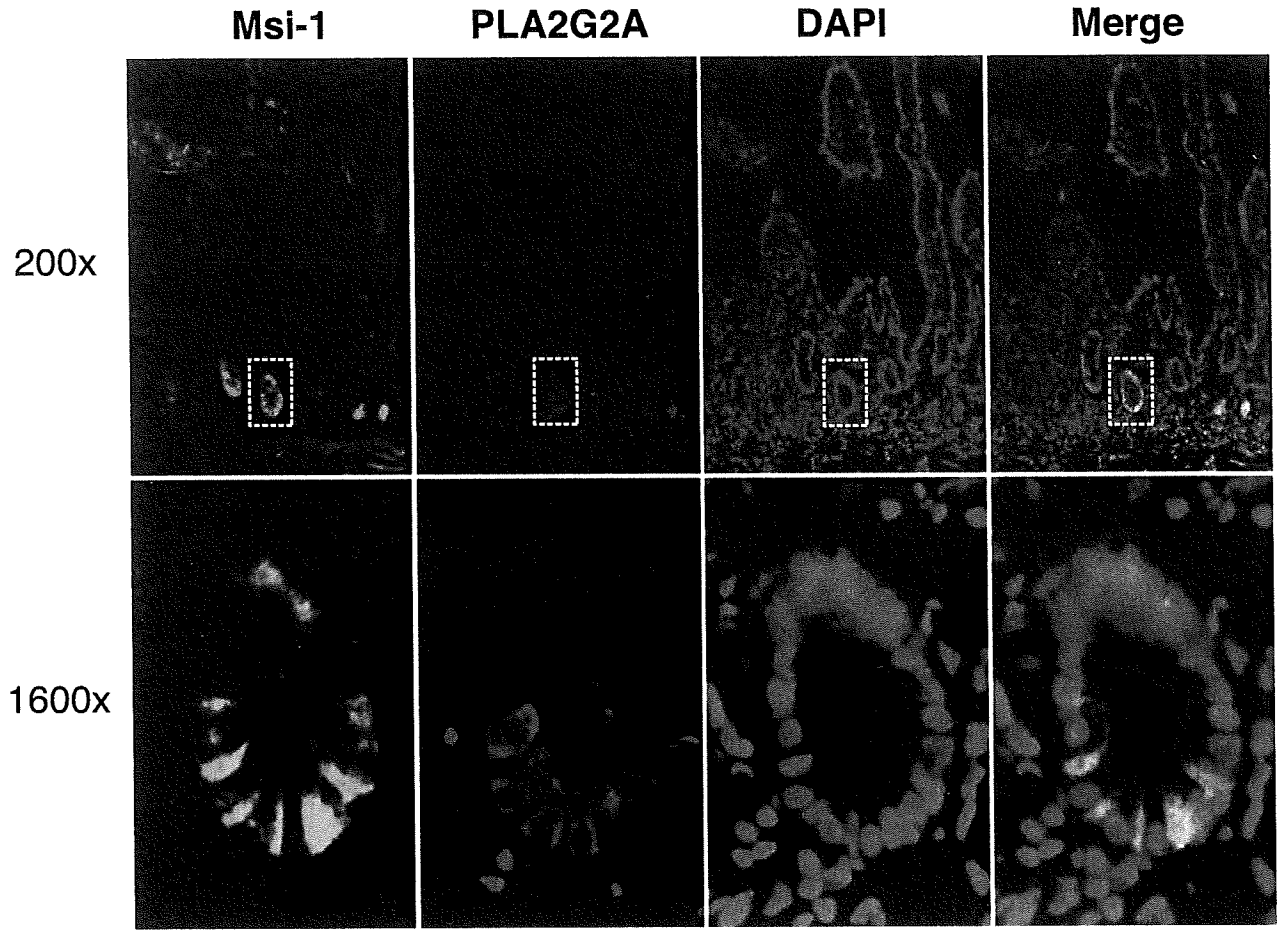

Fig. 5. Distinct cell population within the human intestinal crypt express Msi-1 or PLA2G2A. Double immunofluorescence staining of Msi-1 and PLA2G2A using a human small intestinal tissue is shown. Positive signals for both Msi-1 (*green*) and PLA2G2A (*red*) are observed in epithelial cells residing at the lowest part of the crypt (*upper panel*). A magnified view (*lower panel*) of the designated area (*white squares, upper panel*) shows positive staining of Msi-1 and PLA2G2A, each in distinct intestinal epithelial cells (IECs); coexpression of both proteins in a single IEC is not observed

Distinct populations of crypt epithelial cells expressed Msi-1 or PLA2G2A in the human intestine

To examine whether such a function of Msi-1 might be prevalent also in vivo, we examined the distribution of Msi-1- and PLA2G2A-expressing IECs within the human intestine. Double immunofluorescence staining using human small intestinal tissue showed both Msi-1- and PLA2G2A-expressing IECs locating at the lowest part of the crypt (Fig. 5, upper series). However, a magnified view showed a clear difference in the distribution of Msi-1- and PLA2G2A-expressing IECs, and not a single cell was found to coexpress both proteins (Fig. 5, lower series). Msi-1 expression appeared to be restricted to a population of IECs called the basal columnar cells, characterized by columnar morphology with a thin nucleus, which correspond to the cells recently determined to be definite ISCs.[6] In contrast, PLA2G2A expression was restricted to square cells with rich cytoplasm and a round nucleus, confirming its expression in mature Paneth cells. These findings show that although IECs expressing Msi-1 or PLA2G2A both clustered at the lowest part of the crypt, they each formed a distinct population of cells that were either extremely undifferentiated or fully mature, respectively. As this distribution of Msi-1 and PLA2G2A expression in the human intestine is fully consistent with our previous results, the result suggests that Msi-1 might function as a negative regulator of PLA2G2A expression also in vivo, and thereby contribute to maintain the undifferentiated state of ISCs.

Discussion

In the present study, we demonstrated that expression of Msi-1 in LS174T cells not only induced changes in

cell morphology but also suppressed expression of Paneth cell-specific genes such as *PLA2G2A*. Msi-1 appeared to downregulate *PLA2G2A* expression at the mRNA level by a molecular mechanism independent of the Notch- and Wnt- pathways. As we found that mature Paneth cells, residing adjacent to putative ISCs in vivo, completely lacked Msi-1 expression, loss of Msi-1 expression might be a key event during the process of Paneth cell differentiation from ISCs.

Although *Msi-1* has been reported to be a putative marker gene for ISCs, its functional importance has remained largely unknown.[8,9] As one of the common features of stem cells is to remain undifferentiated, suppression of IEC differentiation could be raised as one possible function of Msi-1. Consistent with this, our present study showed a significant decrease of Paneth cell-specific genes upon Msi-1 expression, suggesting that Msi-1 might function as a negative regulator for IEC differentiation. However, no effect was observed upon genes specific to other lineages, such as *MUC-2* (Fig. 2B). These results may further confirm that maintenance of undifferentiated state in ISCs requires involvement of multiple signaling pathways and molecules, including Msi-1.

Concerning the mechanism of Paneth cell differentiation, previous studies have shown that molecular pathways such as Wnt, Notch, and PPAR-β/δ play critical roles in the development of murine Paneth cells.[7,18,21–23] From our present study, however, the molecular mechanism by which Msi-1 suppresses Paneth cell maturation remains unclear, as it appeared to function independently from both the Notch and Wnt pathways. Further analysis of the Msi-1 target gene within IECs may elucidate the molecular function of Msi-1 in Paneth cell maturation.

The importance of the present finding in vivo may be found in the lowest part of the crypt, where two distinct populations of Wnt-activated IECs reside: ISCs and Paneth cells. Although both types of cell express target genes of the canonical Wnt pathway, they are clearly different in terms of differentiation, as one remains extremely undifferentiated while the other is fully mature. This difference may be, at least in part, mediated by expression of Msi-1, through its function of suppressing Paneth cell differentiation. Thus, our results suggest that expression of Msi-1 in Wnt-activated ISCs may be critically required to avoid progression of the differentiation program toward Paneth cells.

In contrast to IEC differentiation, a series of studies have previously described the role of Msi-1 on IEC proliferation. A recent study using gene knockdown of *Msi-1* in colon cancer cells investigated its role in promoting cell proliferation.[24] Also, another study has reported that p21/CIP1/WAF1 is one of the direct targets of Msi-1.[25] Our present study, however, failed to prove increased cell proliferation upon expression of Msi-1 (Fig. 2A). This failure may due to the aberrant activation of the Wnt pathway in LS174T cells, as this pathway is well known to function as a strong promoter of cell proliferation.[26] Although we were able to observe a two- to threefold increase of TOP-Luc activity upon Msi-1 expression, it appeared to have no significant effect on target genes of the canonical Wnt pathway, thus suggesting that the Wnt signaling pathway might be already fully activated, and no additional function could be achieved in LS174T cells.

Another surprising effect of Msi-1 expression was the significant change in cell morphology and colony formation (Fig. 1C). LS174T cells usually grow in an aggregated form and show a piled-up colony of cells. LS174T/Msi-1 cells, however, grew as a flat monolayer and never formed a colony of piled-up cells. These observations suggested that Msi-1 might also regulate arrangement of the cell cytoskeleton, thereby modulating cell–cell contact or cell motility. Such issues remain to be elucidated in future studies.

In conclusion, Msi-1 suppressed expression of Paneth cell-specific genes, including *PLA2G2A*, in IECs. These findings not only suggest that Msi-1 is a negative regulator of Paneth cell differentiation but also provide insight into functional aspects of Msi-1 expression within ISCs.

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Inhibition of intracellular hepatitis C virus replication by nelfinavir and synergistic effect with interferon-a

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SUMMARY. Liver diseases associated with hepatitis C virus (HCV) infection have become the major cause of mortality in patients with human immunodeficiency virus (HIV) infection since the introduction of highly active anti-retroviral therapy. HCV-related liver disease is more severe in HIV-infected patients than in non-HIV-infected patients, but the standard therapies used to treat chronic hepatitis C in HCV/HIV coinfected patients are the same as those for patients infected with HCV alone. HIV protease inhibitors might have potential to down-regulate HCV load of HCV/HIV coinfected patients. In this study, we evaluated the effects of nelfinavir on intracellular HCV replication using the HCV replicon system. We constructed an HCV replicon expressing a neomycin-selectable chimeric firefly luciferase reporter protein. Cytotoxicity and apoptosis induced by nelfinavir were assessed and synergism between nelfinavir and interferon (IFN) was calculated using CalcuSyn analysis. Nelfinavir dose-dependently repressed HCV replication at low concentrations (IC_{50}, 9.88 1mol/L). Nelfinavir failed to induce cytotoxicity or apoptosis at concentrations that inhibited HCV replication. Clinical concentrations of nelfinavir (5 1mol/L) combined with IFN showed synergistic inhibition of HCV replication in our replicon model. Our results suggest that the direct effects of nelfinavir on the HCV subgenome and its synergism with IFN could improve clinical responses to IFN therapy in HCV/HIV coinfected patients.

Keywords: hepatitis C virus, human immunodeficiency virus, nelfinavir.

INTRODUCTION

Patients with human immunodeficiency virus (HIV) infection are frequently coinfected with hepatitis C virus (HCV), because these viruses have similar routes of transmission, including blood transfusion, intravenous drug use and sexual contact [1,2]. The optimal therapy for HIV infection is highly active anti-retroviral therapy (HAART), which combines HIV reverse transcriptase inhibitors, often with HIV protease inhibitors. Since the introduction of HAART, the morbidity and mortality associated with HIV infection have declined. This reduction in mortality due to opportunistic infections has made HCV-associated liver disease the leading causes of mortality [3].

Several studies have reported that HCV-related liver disease is more severe in HIV-infected patients than in non-HIV-infected patients [4,5]. The severity of liver disease increases as the immunodeficiency progresses and HIV seropositivity accelerates the progression of liver fibrosis related to chronic hepatitis C [6,7]. In addition, many studies have documented that HIV/HCV coinfected patients have higher HCV loads than do HCV monoinfected controls [8–10]. However, the standard therapies used to treat chronic hepatitis C in HCV/HIV coinfected patients are the same as those for patients infected with HCV alone [11].

HAART has been reported to reduce serum HCV RNA levels accompanied by immune improvement [12], but the decrease in HIV viral load was associated with a persistent and significant increase in HCV viral load [13]. There is no consistent evidence that HAART results in suppression of HCV viraemia, suggesting that multiple factors may be affecting viral load in coinfected patients [14,15]. However,

Abbreviations: CI, combination index; HAART, highly active anti-retroviral therapy; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IFN, interferon; LDH, lactate dehydrogenase; MTS, 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl) tetrazolium inner salt; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labelling.

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Trimoulet *et al.* showed that patients treated with HAART that included protease inhibitors had significantly lower intrahepatic HCV loads than those treated with HAART without protease inhibitors [16].

Liver injury has been reported to be a potential side-effect of HAART [17] and potential hepatotoxicity of HIV protease inhibitors had been well realized before these drugs were licensed for the first time [18]. Concomitant hepatic damage prior to the start of HAART is an important risk factor, which can intensify hepatotoxic side-effects of HAART. The presence of chronic hepatitis C has been reported to increase the risk of HAART-associated hepatotoxicity (relative risk, 2.46; 95% confidence interval, 1.43–4.24) [19]. The mechanisms underlying the association of HCV and hepatotoxicity remain unclear, but in some patients liver enzyme elevations may be a manifestation of immune reconstitution that follows antiretroviral therapy After immune recovery, CD4+ cell counts rise and the ability of T cells to identify and lyse HCV-infected hepatocytes may be increased [20]. The differences in the potential for hepatotoxicity have been reported to exist among the commercially available protease inhibitors and nelfinavir was associated with the low rate of severe hepatotoxicity among patients coinfected with hepatitis viruses [21].

HIV protease is a small, dimeric aspartyl protease that specifically cleaves the polyprotein precursors encoding the structural proteins and enzymes of the virus. This proteolytic activity is absolutely required for the production of mature, infectious virions. HIV protease inhibitors block HIV maturation and show remarkable antiviral potency [22]. It has recently been reported that HIV protease inhibitors also have nonviral effects on the host cells, beyond their effect of blocking HIV protease enzymatic activity [23]. NF-jB is central to the overall immune response through its ability to activate genes coding for regulators of apoptosis and cell proliferation [24]. The HIV protease inhibitor nelfinavir has been shown to regulate NF-jB activation [25].

In the present study, we investigated the action of nelfinavir alone, or in combination with interferon (IFN), on HCV replication using the replicon system.

MATERIALS AND METHODS

Cell culture

The human hepatoma cell line, Huh7, was maintained in Dulbecco's modified minimal essential medium (Sigma, St Louis, MO, USA) supplemented with 2 mM L-glutamine and 10% fetal calf serum at 37 °C under 5% CO_2. Huh7 cells expressing the HCV replicon were cultured in medium containing 500 1g/mL G418 (Nakalai Tesque, Kyoto, Japan).

HCV replicon constructs and transfected cell lines

An HCV subgenomic replicon plasmid, pHCVIbneo-delS (designated pRep-N), was derived from an infectious HCV clone, HCV-N, genotype Ib [26]. The replicon, pRep-N was reconstructed by substituting the neomycin phosphotransferase gene with a fusion gene comprising firefly luciferase and neomycin phosphotransferase (pRep-Feo) [27–29]. RNA was synthesized from pRep-Feo using T7-polymerase (Promega, Madison, WI, USA) and transfected into Huh7 cells. After culture in the presence of G418, cell lines stably expressing the replicon were established. We have previously reported that firefly luciferase activities of Feo replicon-expressing cells correlated well with HCV NS3, NS4A and NS5A protein expression levels and with replicon RNA expression levels [27].

Treatment with IFN and nelfinavir

Recombinant human IFN-a-2b (Schering-Plough, Kenilworth, NJ, USA) and purified nelfinavir (Japan Tobacco Inc., Tokyo, Japan) were used. IFN and nelfinavir treatment schedules were as described in the results.

Luciferase assays

Luciferase activity was quantified using a luminometer (Lumat LB9501; Promega) and the Bright-Glo Luciferase Assay System (Promega). Typically, 5×10^3 cells/well, plated onto 24-well plates and cultured for 48 h, were lysed with 100 1L 1× Glo luciferase Buffer (Promega), and the luciferase activity in 100 1L of the lysate was measured by adding an equal volume of Bright-Glo Luciferase Assay Reagent (Promega). Assays were performed in triplicate, and the results were expressed as mean ± SD relative light units.

Western blot analysis

Cells were lysed in buffer containing 62.5 1M Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 6% 2-mercaptoethanol and 0.01% bromophenol blue. Equal amounts of protein (10 1g) were subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (Invitrogen, Carlsbad, CA, USA), followed by transfer to a polyvinylidene difluoride membrane (Roche, Basle, Switzerland) and sequential probing with a monoclonal anti-NS5A antibody (Virogen, Watertown, MA, USA) and b-actin antibody (Thermo Fisher Scientific, Fremont, CA, USA), respectively. The bands were visualized using an enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ, USA).

Cytotoxicity assay

Lactate dehydrogenase (LDH) tests and 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl)tetrazolium inner salt (MTS) reduction assays were performed to investigate cytotoxicity and cell viability. LDH levels were measured in the supernatants using the LDH-Cytotoxic Test (Wako Pure Chemical Industries, Osaka, Japan), according

to the manufacturer's instructions. The level of specific cytotoxicity was calculated using the following formula: % of specific LDH release = ([experimental LDH release – the mean of negative control release]/[the mean of positive control release – the mean of negative control release]) ×100. LDH release from cells treated with 0.2% Tween 20 was used as a positive control, while LDH release from nontreated cells was used as a negative control. Viable cell growth was determined by MTS assay using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega), according to the manufacturer's instructions.

TUNEL method

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labelling (TUNEL) was used to detect DNA fragmentation of nuclei. Using 24-well plates, 5×10^3 cells/well were plated with 5.0 IM nelfinavir. After incubation for 2 days, the glass coverslips were harvested, fixed with 4% paraformaldehyde and washed with phosphate-buffered saline. The cells were permeabilized with 0.5% Tween 20 and treated with MEBSTAIN Apoptosis Kit Direct (Medical and Biological Laboratories Co., Nagoya, Japan). Cells were then treated with RNase and propidium iodide. The nick end-labelling was analysed using a confocal laser scanning microscope (Fluorview; Olympus, Tokyo, Japan).

Analysis of drug synergism

The effects of treatment of Huh7/Rep-Feo cells with nelfinavir and IFN, alone and in combination, were analysed using isobologram analysis. Dose-inhibition curves were drawn for IFN and nelfinavir, used alone or in combination. For each drug combination, the 50% inhibitory concentration (IC_{50}) values were plotted against the fractional concentration of IFN and nelfinavir on the x axis and y axis, respectively. IC_{50}, IC_{20} and IC_{80} values ware determined using the Calcusyn™ software package (Biosoft, Cambridge, UK), which performs single and multiple drug dose-effect calculations and determines the presence of antagonism, additivity or synergism. Using the median effect equation, we used this program to plot dose-effect curves for each drug and combination of drugs. The x intercept of the median effect equation gives the ID_{50} for each drug. The median effect plot also gives information on the slope of the dose-effect curve. This information can then be used to calculate the combination index (CI). CI >1 denotes antagonism, CI = 1 denotes additivity, and CI < 1 denotes synergism.

Statistical analysis

Statistical analysis was performed using the Student's t-test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Effect of nelfinavir on HCV replication

To assess the effects of nelfinavir on intracellular replication of the HCV genome, Huh7/Rep-Feo cells were cultured with various concentrations of nelfinavir. The dose-effect correlation and time course of replicon expression were measured using the luciferase assay 48 h after treatment. Culture of Huh7/Rep-Feo cells with nelfinavir, at concentrations ranging from 0 to 10 1mol/L, showed dose-dependent repression of internal luciferase activity (Fig. 1a). The inhibition of HCV-RNA replication was detectable at concentrations of nelfinavir as low as 2.5 1mol/mL. Western blot hybridization also demonstrated a reduction of the replicon protein levels after nelfinavir treatment (Fig. 1b). To determine the cytotoxic effect of nelfinavir in Huh7/Rep-Feo cells, LDH levels in the supernatants were measured. No significant change in LDH levels was detectable after 48 h incubation (Fig. 1c). MTS assays of the cells cultured with nelfinavir indicated no significant effects on cell viability (Fig. 1d). Nuclear DNA fragmentation in Huh7/Rep-Feo cells, a possible mechanism of nelfinavir induced cytotoxicity, was evaluated by TUNEL staining. No fragmentation of nuclear DNA was observed in Huh7/Rep-Feo cells treated with 5.0 IM nelfinavir (Fig. 1e).

Inhibition of HCV RNA replication by IFN alone and in combination with nelfinavir

Huh7/Rep-Feo cells were cultured with various concentrations of IFN, and the dose-effect correlation and time courses of replicon expression were measured by luciferase assay. IFN caused a marked dose-dependent inhibition of HCV RNA replication (Fig. 2a). The inhibition of HCV RNA replication was detectable at concentrations of IFN as low as 0.01 U/mL. In contrast, measurement of LDH levels and the results of the MTS assay suggested that IFN had little effect on cell viability and replication (data not shown). A dose-effect curve for the effects of nelfinavir and IFN on the replicon was generated by treating Huh7/Rep-Feo cells with various concentrations of IFN (1.0, 0.1, 0.01, 0.001 and 0 U/mL) and nelfinavir (5, 10 and 0 1mol/L). The luciferase activities were plotted against the drug concentrations after 48 h incubation. The inhibition curves were shifted to the left with increasing concentrations of nelfinavir (Fig. 2b), demonstrating synergy between the two drugs against the HCV replicon. There were no significant differences in MTS reduction values at the different drug concentrations (data not shown).

Synergistic inhibitory effects of nelfinavir and IFN on the replicon

We investigated a possible synergistic anti-HCV effect between nelfinavir and IFN, using the isobologram method

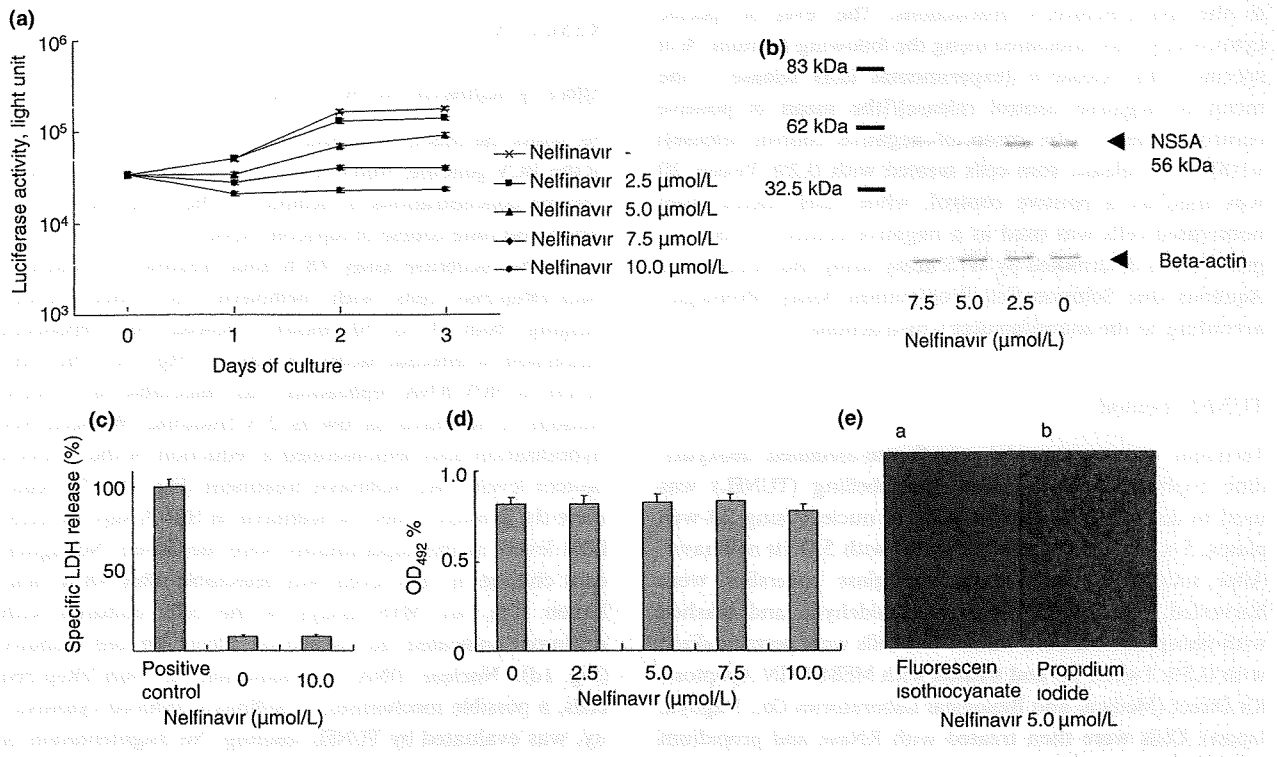

Fig. 1 Dose-dependent inhibition of hepatitis C virus (HCV) RNA replication by nelfinavir. (a) Huh7/Rep-Feo cells were cultured with concentrations of nelfinavir as indicated. (b) Western blotting. The cells were cultured in the presence of nelfinavir, as indicated and were harvested after 48 h exposure. (c) Cytotoxicity assay. Lactate dehydrogenase (LDH) assay of Huh7/Rep-Feo cells cultured with the concentrations of nelfinavir indicated. (d) 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl) tetrazolium inner salt (MTS) assay of Huh7/Rep-Feo cells cultured with the concentrations of nelfinavir indicated. (e) Nuclear DNA fragmentation in Huh7/Rep-Feo cells detected by the TUNEL method. Cells were observed using a confocal laser scanning microscopy (all 200×). Nuclear DNA fragmentation is shown in green (a: fluorescein isothiocyanate staining), and Huh7/Rep-Feo cell nuclei in red (b: propidium iodide staining).

and Calcusyn software, as described in Material and methods. A log dose-effect curve and median effect plot were made for both drugs. Both drugs showed linear regression of effect on the logarithms of doses ($R^2 = 0.94$ for nelfinavir; $R^2 = 0.99$ for IFN). The IC_{50} values were 9.88 ± 0.43 1mol/L for nelfinavir and 0.099 ± 0.14 U/mL for IFN (Fig. 3a,b).

In order to determine if nelfinavir and IFN had a synergistic inhibitory effect on the replicon, Huh7/Rep-Feo cells were treated with combinations of IFN and nelfinavir at various concentrations. Isobolograms were generated based on the two drugs administered in combination at fixed ratios, adjusted for the IC_{50} of each drug (FIC ratio): 1:0, 4:1, 1:1, 1:4 and 0:1. Using the Calcusyn software, each 90% inhibition of HCV replication (Fa = 0.90), 75% inhibition of HCV replication (Fa = 0.75), and 50% inhibition of HCV replication were plotted on the x and y axes (Fig. 3c). The ED_{90}, ED_{75} and ED_{50} plots for each drug ratio fell below the line representing additivity, indicating synergistic effects of the drug combination on intracellular HCV-RNA replication. The CI at an Fa value of 0.5 was 0.58, generated from Fig. 3c using CalcuSyn. There was no significant difference in MTS reduction at different drug concentrations (data not shown), suggesting that the synergistic action of nelfinavir and IFN on HCV replication was through their pharmacological effects, and not due to augmentation of cytotoxicity

DISCUSSION

The results of this study suggest that nelfinavir inhibits HCV replication at concentrations that show no cytotoxicity, and that nelfinavir and IFN act synergistically against HCV.

Nelfinavir inhibited HCV replication in a concentration-dependent manner and its effects could be observed at concentrations as low as <3.0 1mol/L. In clinical use, the plasma concentration of nelfinavir ranges from 3.3 to 6.0 1mol/L. These results support those of Trimoulet *et al.*, who found a reduction in HCV loads in patients treated with HAART including nelfinavir [16]. García-Samaniego *et al.* reported that indinavir, another HIV protease inhibitor, failed to reduce HCV viral titres [4]. In a preliminary study using the replicon system, we tested the ability of several unpurified HIV protease inhibitors to inhibit HCV replication: nelfinavir, ritonavir and saquinavir reduced HCV-replication, but indinavir and fosamprenavir had no effect (data not

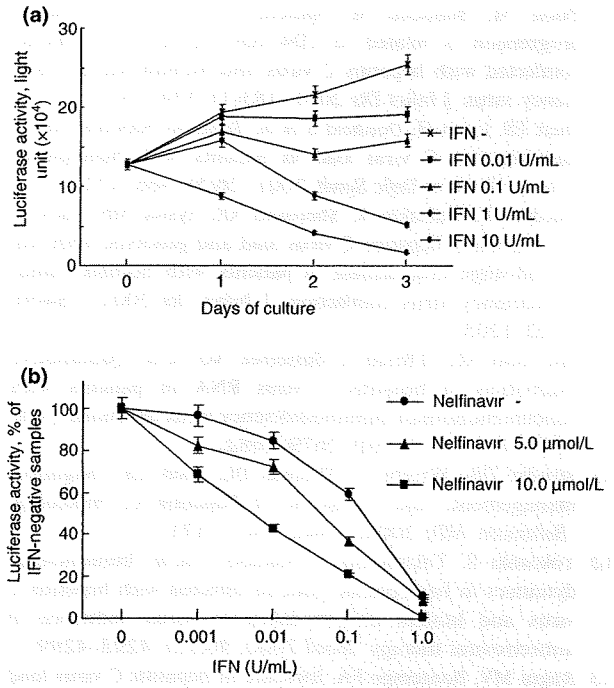

Fig. 2 Dose-dependent inhibition of hepatitis C virus (HCV) RNA replication by IFN. (a) Huh7/Rep-Feo cells were cultured with concentrations of IFN as indicated. (b) Dose-inhibition curves for IFN combined with the concentrations of nelfinavir indicated. Luciferase activities are displayed as percentages of the IFN-negative samples.

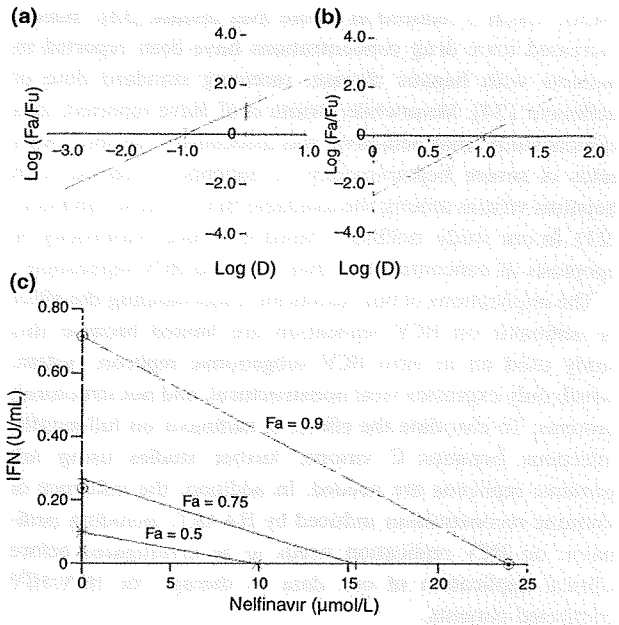

Fig. 3 CalcuSyn analysis of the interferon (IFN)/nelfinavir combination effects on intracellular hepatitis C virus (HCV) replication. Huh7/Rep-Feo cells were cultured with various concentrations of nelfinavir and IFN. Luciferase activities of the cell lysates were measured after 48 h exposure. The CalcuSyn median-effect plot was generated from three separate experiments in triplicate with SD <20% (Fa: affected fraction, Fu: unaffected fraction. D: concentration of drug used). (a) Log dose-effect curve and median effect plot for nelfinavir. Median effect plot has the form of a straight line, $y = 2.47x + 0.43$. (b) Log dose-effect curve and median effect plot for IFN. Median effect plot has the form of a straight line, $y = 1.12x - 0.14$. (c) Isobologram analysis of the combination of IFN and nelfinavir in Huh7/Rep-Feo cells. The individual doses of IFN and nelfinavir required to achieve 90% inhibition of HCV-replication (Fa = 0.90), 75% inhibition of HCV-replication (Fa = 0.75), 50% inhibition of HCV-replication were plotted on the x and y axes. Combination index (CI) values calculated using the Calcusyn software are represented by points above (indicate antagonism between drugs) or below the lines (indicate synergy). (X symbol) ED50, (plus sign) ED75 and (open dotted circle) ED90.

shown). These discrepancies in the effects of different protease inhibitors could explain the different results found in clinical studies.

Combination therapy using ribavirin and IFN is a standard therapy for patients with chronic HCV infection, including HCV/HIV coinfected patients [11]. We previously reported that the use of a clinically achievable concentration of ribavirin, in combination with IFN, showed strong synergistic inhibitory effects on HCV replication using the replicon system [29]. In this study, nelfinavir showed similar strong synergy with IFN. These results suggest that nelfinavir could improve the antiviral effects of IFN in HCV/HIV coinfected patients.

HIV protease inhibitors have a strong affinity for the active site of the HIV viral aspartyl protease, and irreversibly inhibit the catalytic activity of the enzyme. However, HIV protease inhibitors are thought not to inhibit HCV viral serine protease. The above-mentioned findings which demonstrate that different HIV protease inhibitors have different effects on HCV replication support this idea because if HIV protease inhibitors inhibited HCV serine protease, then all HIV protease inhibitors should inhibit HCV replication. The mechanism by which nelfinavir inhibits HCV replication is uncertain: Several studies have shown that HCV infection alters NF-jB promoter activity, possibly contributing to the persistence of HCV infection [30–32]. Equils *et al.* reported that nelfinavir blocked TLR2-, TLR4- and TNF-a-induced NF-jB activation [25]. Nelfinavir may play an important role in the regulation of the cellular inflammatory and immune responses through NF-jB, but further studies are needed to investigate the role of NF-jB promoter activity in nelfinavir-induced HCV replication inhibition.

The decreased clearance of antiretroviral drugs is suspected to be a possible cause of increased susceptibility for HAART-associated liver toxicity in HIV/HCV coinfected patients, because the metabolism of the HIV protease inhibitors depends on the amount of functional cytochrome

P450, which is reduced in severe liver disease [33]. Indeed, increased toxic drug concentrations have been reported in patients with hepatic disease, receiving standard dose of nelfinavir [34]. Meanwhile, Bruno *et al.* have reported, in a clinical study, that nelfinavir was associated with the lowest rates of severe hepatotoxicity in patients coinfected with hepatitis viruses among the available HIV protease inhibitors [21]. In our study, nelfinavir failed to induce cytotoxicity or apoptosis at concentrations that inhibited HCV replication.

The implications of our results for understanding the effect of nelfinavir on HCV replication are limited because this study used an *in vitro* HCV subgenomic replicon system, which only expresses viral nonstructural, and not structural, proteins. To elucidate the effects of nelfinavir on full-length, infectious hepatitis C virions, further studies using full genomic replicons are needed. In addition, the influence of immune reconstitution induced by HAART, including nelfinavir, on HCV replication needs to be investigated before clinical application of our data to therapy for HCV/HIV coinfected patients.

Because end-stage chronic liver disease resulting from co-infection with HCV is now the major cause of death in individuals infected with HIV, our results suggest a potentially promising approach for improving the standard therapies for chronic hepatitis C in HCV/HIV coinfected patients.

CONFLICT OF INTEREST

The authors report no conflict of interest.

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Curcumin inhibits hepatitis C virus replication via suppressing the Akt-SREBP-1 pathway

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ABSTRACT

A polyphenolic compound from the curry spice turmeric, curcumin, is known to show anti-viral activity against the influenza virus, adenovirus, coxsackievirus, and the human immunodeficiency virus. However, it remains to be determined whether curcumin can inhibit the replication of hepatitis C virus (HCV). In this study, we showed that curcumin decreases HCV gene expression via suppression of the Akt-SREBP-1 activation, not by NF-κB pathway. The combination of curcumin and IFNα exerted profound inhibitory effects on HCV replication. Collectively, our results indicate that curcumin can suppress HCV replication in vitro and may be potentially useful as novel anti-HCV reagents.

1. Introduction

The hepatitis C virus (HCV), a member of the *Flaviviridae* family, is an enveloped virus with a single-stranded 9.6 kb RNA genome. HCV infection is characterized by a high rate of progression to fibrosis and chronic hepatitis, resulting in cirrhosis, and ultimately in hepatocellular carcinoma [1]. The best anti-viral therapy presently known involves the combination of pegylated interferon (IFN) alpha and ribavirin, but almost half of all patients manifest no response to exogenous IFNα [2]. Therefore, the development of novel drugs for the safer and more efficient treatment of HCV is urgently required.

Many bioactive polyphenolic compounds have been shown to perform candidate agent functions in chemoprevention and in cancer chemotherapy [3]. Among this class, curcumin (diferuloylmethane) is one of the most widely studied compounds. Curcumin is the major component of the curry spice tumeric (*Curcuma longa* Linn) and can affect the metabolism of cells and organisms in a number of ways, including anti-inflammatory, anti-oxidant, and anti-proliferative properties via the modulation of multiple cellular mechanisms [4,5]. Furthermore, some recent reports have shown that these compounds show anti-viral activity against the influenza virus, adenovirus, coxsackievirus, and the human immunodeficiency virus [6–9]. Also, curcumin has been shown to suppress transcription activation by the host protein AP-1, leading to diminished HTLV-1 and HPV-mediated cellular transformation [10]. However, it remains to be determined whether curcumin can inhibit the gene expression of HCV

On the basis of our previous knowledge of the regulation of HCV replication and the biological properties of curcumin, we evaluated the effects of curcumin on the intracellular replication of the HCV genome in vitro, using an HCV replicon system. We showed that curcumin at concentrations that do not affect cell viability reduced HCV RNA replication in vitro to a significant degree. Curcumin inhibited a lipogenic transcription factor, sterol regulatory element binding protein-1 (SREBP-1)-induced HCV replication via the PI3K/Akt pathway. Finally, the combination of curcumin and IFNα showed cooperative inhibitory effects on HCV RNA replication. Our results indicate that curcumin may potentially prove useful as a treatment for HCV infection.

2. Materials and methods

2.1. Plasmid constructs

pEMCV/IRES-Rluc was utilized as a control for the analysis of translation efficiency mediated by an encephalomyocarditis virus internal ribosome entry site (EMCV-IRES) which mediates the translation of the HCV non-structure gene of replicon constructs, Huh7/Rep-Feo [11]. pCIneo-Rluc-IRES-Fluc was constructed in or-

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der to evaluate HCV internal ribosome entry site (IRES)-mediated translation efficiency [12]. The plasmid expressed a bicistronic RNA, in which Rluc was translated in a cap-dependent manner and Fluc was translated via HCV IRES-mediated initiation.

2.2. Cell cultures and treatments

Huh7 cells expressing the HCV replicon (Huh7/Rep-Feo) were maintained in DMEM supplemented with 10% FBS containing 500 μg/ml of G418 (Calbiochem). Huh7/Rep-Feo and Huh7 cells were plated at 70–80% confluence and treated with various concentrations of curcumin or vehicle controls and incubated for 24 h at 37 °C. The concentration ranges of 5–15 mM curcumin (obtained from Sigma) were tested. Control vehicle treatment (DMSO) was equivalent to the highest concentrations in the dose range experiments for each of the tested drugs.

2.3. HCV replicons

An HCV subgenomic replicon plasmid, pRep-Feo (Fig. 1A), was derived from pRep-Neo (originally referred to as pHCVIbneo-delS) [13]. Replicon RNA was synthesized in vitro using T7-RNA polymerase (Promega, Madison, WI) and transfected into the Huh7 cells via electroporation. After culturing in the presence of G418, the cell lines stably expressing the replicons were established and designated Huh7/Rep-Feo.

2.4. Transient transfection and luciferase reporter assay

Plasmid transfection was conducted using PolyFect (QIAGEN) in accordance with the manufacturer's instructions. The pcDNA3.1 empty vector was added to the transfections in order to achieve the same total amount of plasmid DNA per transfection. The cells were lysed in cell culture lysis buffer (Promega). The luciferase activity was evaluated using an analytical luminescence luminometer in accordance with the manufacturer's instructions.

2.5. MTT assay

For cell viability assay, Huh7/Rep-Feo cells were seeded in a 24-well tissue culture plate and incubated for 24 h. Cells were treated with curcumin or Bay11-7082. After 24 h, MTT (3-(4,5-dimethylthiozol-2-ly)-2,5-diphenyltetrazolium bromide) solution (0.5 mg/ml) was added to each well. After incubation for 2 h at 37 °C, formazan crystals in viable cells were soluble in 200 μl of DMSO. The soluble formazan product was spectrophotometrically quantified using an ELISA leader at 570 nm.

2.6. siRNA design and siRNA transfection

RNA oligonucleotides were synthesized by Bioneer (Daejeon, Republic of Korea). The sequences of siRNA targeting human p65 were sense, 5′-GAU UGA GGA GAA ACG UAA A-3′ and antisense, 5′-UUU ACG UUU CUC CUC AAU C-3′ The sequence of siRNA targeting human SREBP-1 were sense, 5′-UGA GUG GCG GAA CCA UCU U-3′ and antisense, 5′-AAG AUG GUU CCG CCA CUC A-3′. The scramble control siRNA sequences were sense, 5′-CCU ACG CCA CCA AUU UCG U-3′ and antisense, 5′-ACG AAA UUG GUG GCG UAG G-3′ The cells were transfected with siRNA using HiPerFect (QIAGEN) according to the instructions of the manufacturer.

2.7 RT-PCR analysis

Total RNA from the curcumin-treated Huh7/Feo cells was prepared using TRIzol reagent (Invitrogen) by following the manufacturer's instructions. cDNA was used as a template for real-time PCR using gene-specific primers: NS3, 5′-TCG TGG CAA CAG ACG CTC TAA TGA-3′ (forward) and 5′-AGA ACT CCA G AT GGT CCT GGC AAA-3′ (reverse); NS5A, 5′-TAG CAG TGC TCA CTT CCA TGC TCA-3′ (forward) and 5′-AGG ATC TCC GCC GCA ATG GAT ATT-3′ (reverse); β-actin, 5′-GAC TAC CTC ATG AAG ATC-3′ (forward), 5′-GAT CCA CAT CTG CTG GAA-3′ (reverse).

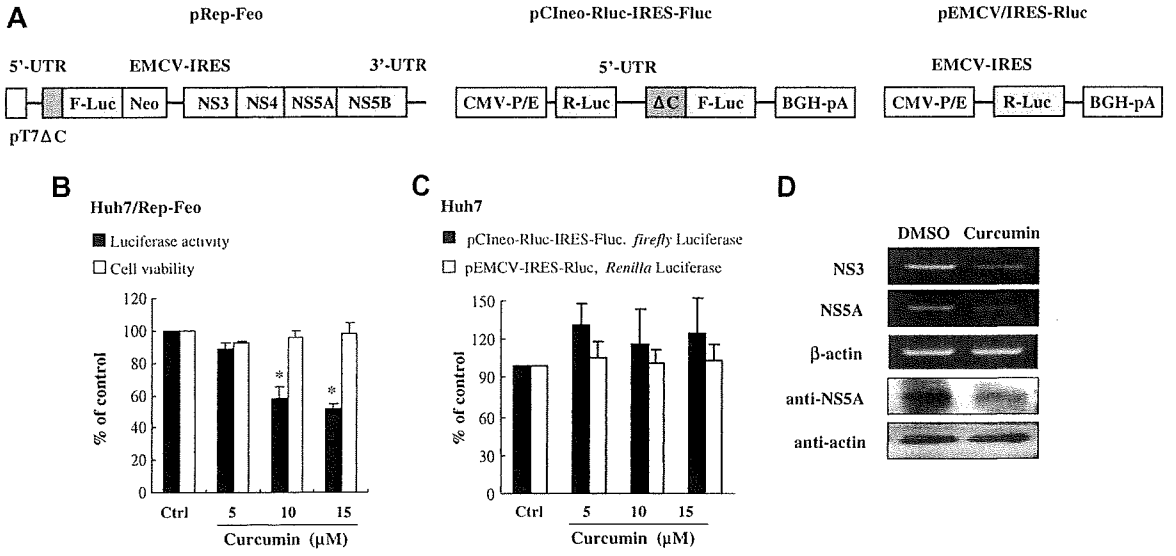

Fig. 1. Curcumin suppresses HCV RNA replication. (A) Structures of the hepatitis C virus (HCV) replicon, pEMCV/IRES-Rluc, and pCIneo-Rluc-IRES-Fluc plasmids. (B) The effects of curcumin on Huh7/Rep-Feo cells on luciferase activity and MTT assays. For the luciferase assay, the Huh7/Rep-Feo cells were cultured in the presence of the indicated curcumin concentrations, and the luciferase activity was determined at 24 h of treatment. The values are expressed as the means ± S.D. for at least three independent experiments. *$P < 0.05$ compared with control. (C) The luciferase activity effects on HCV IRES-mediated translation and EMCV/IRES-mediated translation by curcumin. The data are expressed as the means ± S.D. ($n = 3$). (D) The inhibitory effect of HCV RNA and protein level by curcumin. Huh7/Rep-Feo cells were cultured for 24 h in the presence of 15 μM curcumin. Total RNA was extracted from the cells, and the levels of NS3 and NS5A mRNA were determined by RT-PCR and β-actin expression is shown as an mRNA-loading control. Total protein extracts were blotted with anti-NS5A antibody and actin expression is shown as a protein-loading control. Three independent experiments were reproduced.

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2.8. Nuclear/cytosolic fractions analysis

Cells were lysed in buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 0.5% NP-40, 1 mM PMSF, protease inhibitors) and incubated for 10 min on ice. The supernatants (cytosolic lysates) were collected by centrifugation (3300×g) at 4 °C for 5 min. The nuclear pellets were then washed with ice-cold PBS to avoid contamination of cytosolic proteins and lysed in buffer B (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 0.5% NP-40, 1 mM PMSF, protease inhibitors). After incubation on ice for 25 min, the supernatants (nuclear lysates) were collected by centrifugation (13 000×g) at 4 °C for 5 min.

2.9. Statistical analysis

Statistical analyses were carried out by unpaired or paired *t* test as appropriate. All data are reported as means ± S.D. *P* value of <0.05 was considered significant.

3. Results

3.1. Curcumin suppresses HCV RNA replication

In order to evaluate the effects of curcumin on the intracellular replication of the HCV genome, Huh7/Rep-Feo cells were treated with various concentrations of curcumin. The luciferase activity of the Huh7/Rep-Feo cells demonstrated that the replication of the HCV replicon was suppressed by curcumin. The MTT assay showed no effects on cell viability at various concentrations of these compounds (Fig. 1B). Moreover, the efficiency of EMCV-IRES-mediated translation was not affected by curcumin (Fig. 1C). These data indicate that the inhibitory effects on HCV replication exerted by curcumin are not attributable to cytotoxicity or to an artificial effect on the EMCV-IRES, which directly translates the HCV non-structure protein of the replicon. We then attempted to determine whether these effects of curcumin on the replication of the HCV replicon are involved in HCV IRES-dependent translation. We determined that curcumin gives no effects on the activity of *Firefly* luciferase in Huh7 cells that were transiently transfected with the pCIneo-Rluc-IRES-Fluc reporter (Fig. 1C). Taken together, these results indicate that curcumin suppresses HCV replication in vitro and that these effects are not involved in cell viability, EMCV-IRES-mediated translation, or HCV IRES-dependent translation.

In an effort to confirm these inhibitory effects on the HCV replicon by curcumin, we attempted to determine whether curcumin affects the HCV RNA and protein level. HCV replicon RNA level was detected by RT-PCR by using primers specific to NS3 and NS5A and protein level was assayed by Western blots with the anti-NS5A antibody. As shown in Fig. 1D, curcumin-treated cells expressed lower levels of the HCV replicon RNA as compared with the control vehicle-treated Huh7/Rep-Feo cells. Also, we showed that curcumin decreases the protein level of HCV NS5A, translated from the

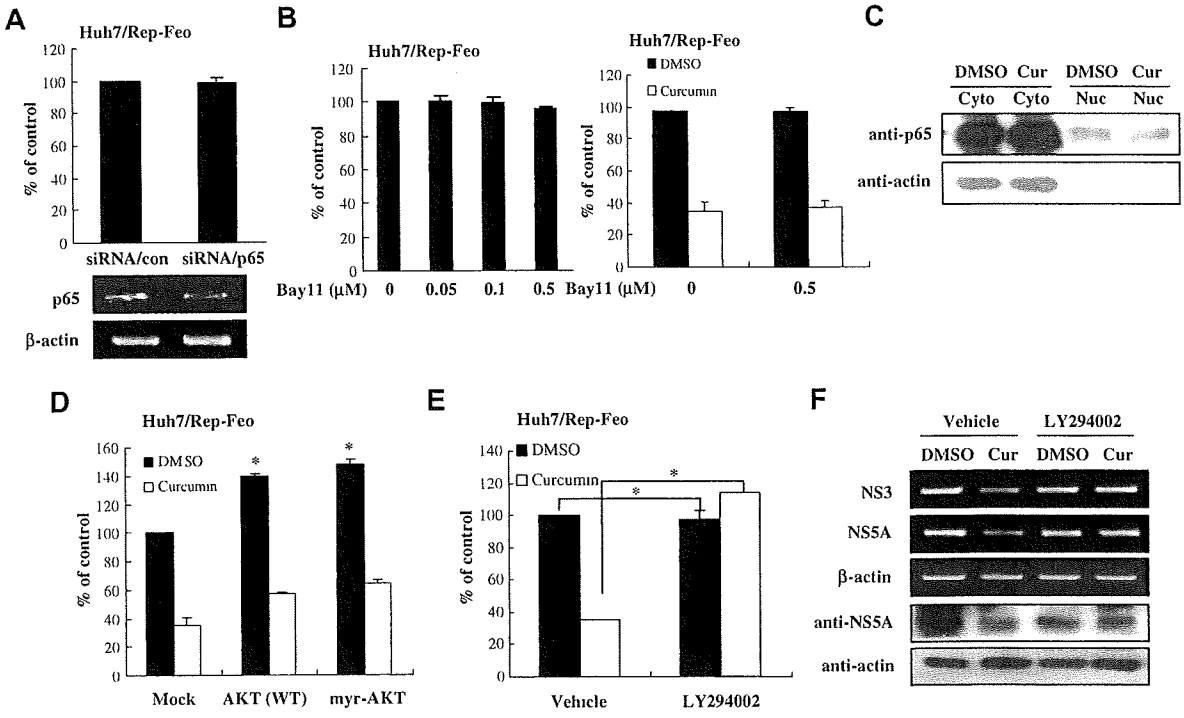

Fig. 2. Curcumin suppressed HCV replication via the PI3 K/Akt pathway. (A) The effects of p65 siRNA on HCV replication. Huh7/Rep-Feo cells were seeded in 6-well culture plates and transfected with scramble control siRNA and p65 siRNA and luciferase activities are measured ($n = 3$). (B) The effects of Bay11-7082 on HCV replication. Huh7/Rep-Feo cells were seeded in 24-well culture plates and treated with the Bay11-7082. The effects of curcumin on Huh7/Rep-Feo cells were identified by MTT assays (left) and luciferase activity (right) ($n = 3$). (C) The effects of curcumin on the nuclear localization of p65 protein. Huh7/Rep-Feo cells were seeded in 6-well culture plates and treated with curcumin (15 μM). The cyto or nuc indicates the cytosolic or nuclear extracts. (D) The effect of Akt on HCV RNA replication. Huh7/Rep-Feo cells were transfected with the plasmid for Akt (WT) or myr-Akt and cells were treated for 24 h with 15 μM curcumin or vehicle (DMSO). Luciferase activity was measured and the values are expressed as the means ± S.D. for at least three independent experiments. (E and F) The effect of LY249002 on curcumin-suppressed HCV RNA replication. Huh7/Rep-Feo cells were pretreated with 25 μM LY294002 for 2 h, and then incubated further in the absence or presence of 15 μM curcumin for 24 h. Luciferase activities were measured ($n = 3$) (E). NS3 or NS5A mRNA levels were detected using RT-PCR and protein levels of NS5A were detected by Western blotting ($n = 2$) (F).

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HCV replicon (Fig. 1D). These data correlate well with the luciferase activity of Huh7/Rep-Feo cells.

3.2. Curcumin inhibits HCV replication via the PI3K/Ak pathway, not NF-κB pathway

In an effort to gain insight into the molecular mechanism by which curcumin suppresses HCV replication, we then analyzed the signaling pathways involved in curcumin-inhibited HCV replication. Previously, it was suggested that the activation of the NF-κB pathway is involved in the increase of HCV replication [14,15]. To evaluate the involvement of NF-κB pathway on the curcumin-mediated inhibition of HCV replication, p65 (of NF-κB) siRNA and Bay11-7082 (a specific inhibitor of NF-κB pathway) were exploited. However, in contrast to our expectations, p65 siRNA and Bay11-7082 had no effect on HCV replication (Fig. 2A and B). Furthermore, curcumin also had no effect on the nuclear localization of p65 (Fig. 2C). These results show that PDTC and curcumin exert a synergic-inhibitory effect on HCV replication via NF-κB-independent pathway.

We next explored other potential pathways for HCV replication by curcumin. Several signaling pathways and transcription factors including AP-1, mitogen-activated protein kinases (MAPKs), and cell cycle machinery have been suggested as the targets of curcumin. We examined the effect of several pathways on HCV replication using treatments of specific kinase inhibitors or transfection of plasmid for specific kinases, including ERK, JNK, p38, Akt, and PKA. Among many signaling pathways, transfection of plasmid for wild type (WT)-Akt or constitutive active (myr)-Akt significantly augmented the luciferase activities of HCV replicon (Fig. 2D). Also, LY294002, a specific inhibitor of the PI3K/Akt pathway, inhibited curcumin-suppressed activity of HCV replicon (Fig. 2E). To confirm these effects on the HCV replicon induced by curcumin, we examined the HCV RNA and protein levels. As shown in Fig. 2F, LY294002-treated cells restrained the RNA and protein levels of HCV replicon inhibited by curcumin. Taken together, these results suggest that curcumin suppresses HCV replicon expression via the PI3K/Akt pathway.

3.3. SREBP-1 increases the HCV replicon expression via the PI3K/Akt pathway

To identify the downstream target of the PI3K/Akt pathway on HCV RNA replication, we tested several transcription factors regulated by the PI3K/Akt pathway. Among them, two SREBP-1 isoforms, SREBP-1a and SREBP-1c, induced the luciferase activity of the Huh7/Rep-Feo cells. Especially, SREBP-1a showed a profound effect on HCV replication (Fig. 3A). Previously, it has been suggested that the activation of Akt was able to increase the SREBP-1 gene expression [16]. Also, in an effort to determine whether SREBP-1 performs a function in HCV RNA replication, we attempted to knockdown SREBP-1 expression using siRNA for SREBP-1. As shown in Fig. 3B, siRNA/SREBP-1-transfected cells decreased the luciferase activity of the Huh7/Rep-Feo cells.

In an effort to confirm these effects on the HCV replicon exerted by SREBP-1, we attempted to determine whether SREBP-1 affects the HCV RNA and protein level. As shown in Fig. 3C, SREBP-1a-transfected cells enhanced the levels of the HCV replicon RNA as

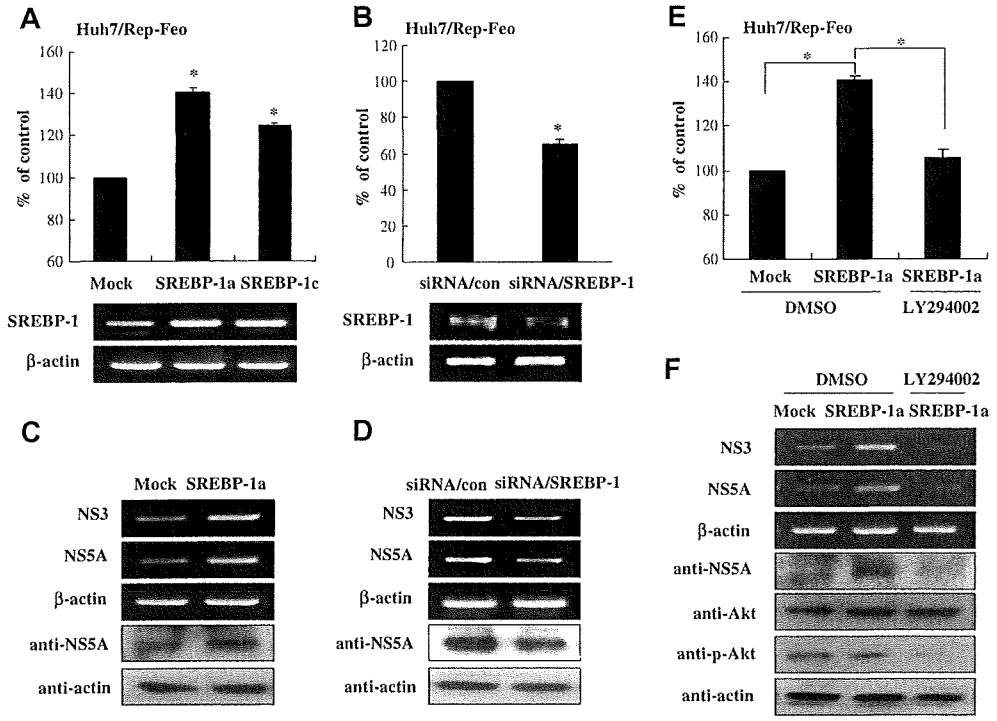

Fig. 3. SREBP-1 increases the HCV replicon expression via the PI3 K/Akt pathway. (A and B) The effects of SREBP-1 on HCV replication. Huh7/Rep-Feo cells were transfected with the plasmid for SREBP-1a or SREBP-1c and luciferase activities were measured ($n = 3$) (A). Huh7/Rep-Feo cells were transfected with scramble control siRNA and SREBP-1 siRNA and luciferase activities were measured ($n = 2$) (B). *$P < 0.05$ compared with control. (C and D) The effects of SREBP-1 on HCV RNA and protein levels. Huh7/Rep-Feo cells were transfected with the plasmid for SREBP-1a (C) or siRNA for SREBP-1 (D). NS3 or NS5A mRNA levels were detected using RT-PCR and protein levels of NS5A were detected by Western blotting ($n = 2$). (E and F) The effect of LY294002 on SREBP-1-induced HCV replication. Huh7/Rep-Feo cells were transfected with the plasmid for SREBP-1a and then incubated further in the absence or presence of 25 μM LY294002 for 24 h. Luciferase activities were measured ($n = 3$) (E) and NS3 or NS5A mRNA and NS5A protein levels were determined using RT-PCR or Western blotting ($n = 2$) (F).

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compared with the control transfected-Huh7/Rep-Feo cells. Also, we showed that the knockdown of SREBP-1 expression decreases the RNA and protein level of HCV replicon (Fig. 3D). Furthermore, SREBP-1a-transfected cells did not induce the HCV RNA replication in the presence of LY294002 (Fig. 3E and F). Taken together, SREBP-1 can increase the HCV replication via the PI3 K/Akt pathway.

3.4. Curcumin suppresses the SREBP-1-induced HCV replication

In order to determine whether SREBP-1-increased HCV RNA replication is inhibited by curcumin, Huh7/Rep-Feo cells were transfected with the plasmid for SREBP-1a in the absence or presence of curcumin treatment. As shown in Fig. 4A, the luciferase activities of Huh7/Rep-Feo cells were inhibited by curcumin in the SREBP-1a transfection. Also, these effects were confirmed on the RNA and protein levels of HCV replicon (Fig. 4B).

3.5. Curcumin and IFNα have synergistic inhibitory effects on HCV replication

Finally, we examined whether curcumin can affect IFNα-based inhibition of HCV IFNα has been shown to exert inhibitory effects on HCV replication. In order to determine whether curcumin could affect the IFNα-mediated inhibitory effect on HCV replicon, Huh7/Rep-Feo cells were treated with a combination of IFNα and curcumin. As shown in Fig. 5A and B, curcumin significantly augmented the IFNα-mediated inhibition of HCV replication. Thus, these findings demonstrate that cotreatment with curcumin was more effective than treatment with IFNα alone.

4. Discussion

There is abundant evidence indicating that dietary phytochemicals, including epigallocatechin gallate (EGCG), curcumin, resveratrol, and genistein, show anti-viral effects in a variety of virus types [6–9,17,18]. However, it remains to be determined whether dietary phytochemicals have anti-viral activity in cases of Hepatitis C. It was reported that curcumin exhibits anti-oxidant activity by reducing the generation of reactive oxygen species (ROS). Although it has been known that oxidative stress decreases HCV replication [19], a recent study showed that ROS is able to increase the replication of HCV [20]. According to this study, anti-oxidant pyrrolidine dithiocarbamate (PDTC) treatment decreased the expression of HCV RNA in Huh7 cells expressing HCV subgenomic replicons. In the present study, we observed that PDTC reduces the activity of HCV luciferase replicons in Huh7/Rep-Feo cells (data not shown). In addition, curcumin suppressed the HCV replication in cooperation with PDTC. Although curcumin had no effect on the

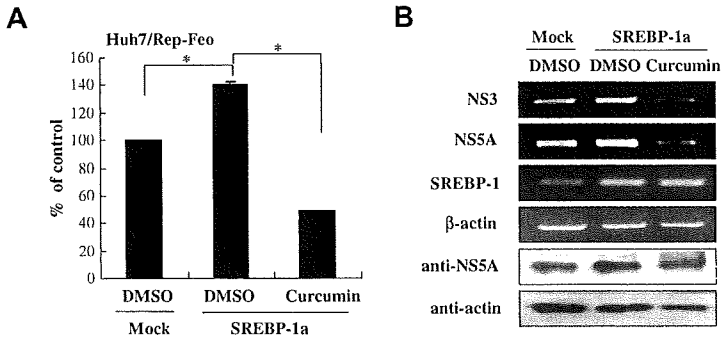

Fig. 4. Curcumin suppresses the SREBP-1-induced HCV replication. (A and B) The effects of SREBP-1 on curcumin-treated HCV replicon. Huh7/Rep-Feo cells were transfected with the plasmid for SREBP-1a and then incubated further with or without 15 μM curcumin for 24 h. Luciferase activities were measured ($n = 3$) (A) and NS3 or NS5A mRNA and NS5A protein levels were determined using RT-PCR or Western blotting ($n = 2$) (B).

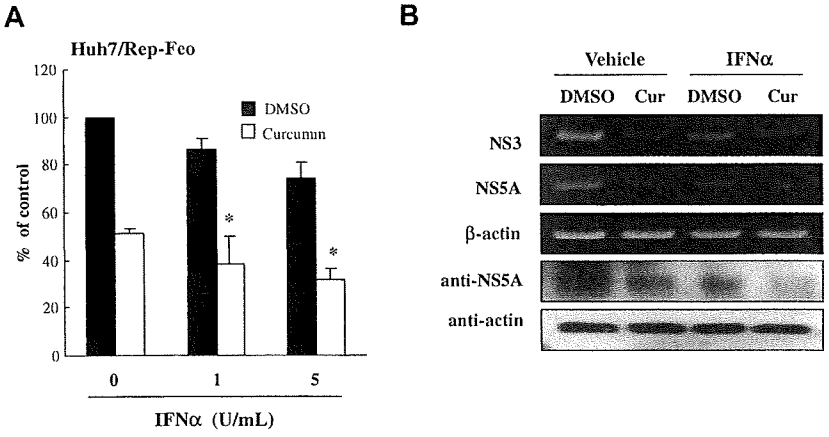

Fig. 5. curcumin and IFNα have synergistic inhibitory effects on HCV replication. (A) Huh7/Rep-Feo cells were cultured in the presence or absence of curcumin (15 μM) and IFNα, (0, 1, 5 U/ml). After 24 h of treatment, the cell lysates were obtained and luciferase activity was measured. The data shown are expressed as the means ± S.D. ($n = 3$). *$P < 0.05$ compared with curcumin alone or IFNα alone. (B) Huh7/Rep-Feo cells were incubated in the presence or absence of curcumin (15 μM) and IFNα (5 U/ml). After 24 h, NS3 or NS5A mRNA levels or NS5A protein levels were detected using RT-PCR or Western blotting ($n = 2$).

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gene expression change of anti-oxidant enzymes, such as, Cu/Zn-superoxide dismutase (Cu/Zn-SOD), Mn-superoxide dismutase (Mn-SOD) (data not shown), because curcumin exert the function as anti-oxidant through various pathways, we could not rule out the possibility that curcumin decreases HCV replication as anti-oxidant.

Some reports were demonstrated that despite its beneficial, direct anti-tumor actions, curcumin (and potentially other natural products) may adversely modulate the cellular response to clinically relevant cytokines or cytotoxic activities against a variety of tumor targets [21,22]. Therefore, curcumin experimentally evaluated has been found to be non-toxic or to have effective doses far below its toxic doses in the cancer therapy or blood lipid profile [23,24]. It is necessary to perform experiments to identify the adequate concentrations of curcumin in relation to inhibition of HCV replication in vivo.

Curcumin is considered to be a potentially important chemopreventive agent against a variety of cancers, including liver cancer [25]. Recently, it has been reported that curcumin inhibits the development of human hepatocellular carcinoma [26,27]. In this report, we demonstrated that curcumin inhibits HCV replicon expression via the PI3K/Akt-SREBP-1 pathway. Taken together with previous findings, although hepatocellular carcinoma is the outcome of complicated processes by various genetic factors and environmental factors, our current data suggests the possibility that curcumin may hinder the development of liver cancer via the inhibition of HCV replication in HCV-induced hepatocellular carcinoma.

Acknowledgments

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Journal of Medical Virology 81:452–458 (2009)

A Matched Case-Controlled Study of 48 and 72 Weeks of Peginterferon Plus Ribavirin Combination Therapy in Patients Infected With HCV Genotype 1b in Japan: Amino Acid Substitutions in HCV Core Region as Predictor of Sustained Virological Response

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Substitution of amino acid (aa) 70 and 91 in the core region of HCV genotype 1b is a useful pretreatment predictor of efficacy of 48-week peginterferon (PEG-IFN) plus ribavirin (RBV) therapy. Here, we determined the efficacy of 72-week PEG-IFN/RBV and the predictive factors to such therapy in a case–control study matched for sex, age, and periods from the start of treatment to initial point of HCV RNA-negative. We compared the treatment efficacy of 72-week regimen in 65 patients with that of 48-week in 130 patients, who were infected with HCV genotype 1b and treated with PEG-IFN/RBV. They consisted mainly of late virological responders (LVR) (HCV RNA-positive at 12 weeks and negative at 24 weeks after start of treatment). Sustained virological response (SVR) was achieved by 61.5% and 32.3% of patients of the 72- and 48-week groups, respectively, while non-virological response was noted in 9.2% and 29.2% of the respective groups. Multivariate analysis identified substitution of aa 70 and 91 (Arg70 and/or Leu91) and duration of treatment (72-week) as independent parameters that significantly influenced SVR. For Arg70 and/or Leu91 of core region, SVR rate was significantly higher in 72- (68.0%) than 48-week group (37.8%). For wild-type of ISDR, SVR rate was significantly higher in 72- (61.2%) than in 48-week group (29.3%). We conclude that 72-week PEG-IFN/RBV improves SVR rate for LVR, especially those with Arg70 and/or Leu91 of core region or wild-type of ISDR. Substitution of aa 70 and 91 is also a useful pretreatment predictor of response to 72-week PEG-IFN/RBV. ***J. Med. Virol. 81:452–458, 2009.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: HCV; core region; NS5A-ISDR; peginterferon; ribavirin; 72-week; case–control study; LVR

INTRODUCTION

Hepatitis C virus (HCV) usually causes chronic infection that can result in chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [Dusheiko, 1998; Ikeda et al., 1998; Niederau et al., 1998; Kenny-Walsh, 1999; Akuta et al., 2001]. In patients with HCV-chronic hepatitis, treatment with interferon (IFN) can induce viral clearance and marked biochemical and histological improvement [Davis et al., 1989; Di Bisceglie et al., 1989]. Especially, peginterferon (PEG-IFN) plus ribavirin (RBV) combination therapy for 48 weeks can achieve a high sustained virological response (SVR) [Manns et al., 2001; Fried et al., 2002].

Although treatment of genotype 1-infected patients typically extends over 48 weeks, there has been interest in prolongation of therapy, particularly in late

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virological responders (LVR) (HCV RNA-positive at 12 weeks and negative at 24 weeks after the start of treatment), because high relapse rates in LVR may indicate that treatment was not administered for a sufficient duration [Ferenci et al., 2005]. Previous studies from Europe and United States have demonstrated that LVR improves SVR rates when treatment is extended to 72 weeks, compared with standard duration of therapy, largely as a result of reducing posttreatment relapse rates [Buti et al., 2003; Berg et al., 2006; Sánchez-Tapias et al., 2006; Pearlman et al., 2007]. Thus, prolongation of therapy in LVR may improve the virological response rate. However, it is not clear at present whether prolongation of treatment improves the SVR rate of treatment-resistant Japanese patients infected with HCV/genotype 1b [Akuta et al., 2007a,b,c].

Previous studies indicated that amino acid (aa) substitutions at position 70 and/or 91 in the HCV core region of genotype 1b were predictors of poor virological response to 48-week PEG-IFN plus RBV therapy [Akuta et al., 2005, 2006, 2007a,b,c; Donlin et al., 2007], and also risk factors for hepatocarcinogenesis [Akuta et al., 2007d, 2008a]. However, it is not clear at this stage whether aa substitutions in the core region can be used before therapy to predict the outcome of 72-week regimen.

The aims of the present study in HCV genotype 1b-infected Japanese adult patients, who received PEG-IFN plus RBV, were the following: (1) To conduct a case–control study matched for sex, age, and periods from the start of treatment to the initial point of HCV RNA-negative, to compare the treatment efficacy of 72-week regimen and 48-week regimen. (2) To identify the pretreatment factors that could predict treatment efficacy of the 72-week regimen, including pretreatment aa substitutions in the core region.

PATIENTS AND METHODS

Study Population

A total of 559 HCV genotype1b-infected Japanese adult patients were consecutively recruited into the study protocol of combination therapy with PEG-IFNα-2b plus RBV between 2001 and 2008 at Toranomon Hospital, Tokyo, Japan. They received PEG-IFNα-2b at a median dose of 1.4 μg/kg (range, 0.7–2.1 μg/kg) subcutaneously each week plus oral RBV at a median dose of 11.1 mg/kg (range, 3.4–16.0 mg/kg) daily. Among these, 383 patients, who could complete a total of 48 or 72 weeks of combination therapy, were enrolled in this retrospective study. The latter group consisted of 65 patients who extended combination therapy to 72-week (72-week group), and 318 patients who stopped combination therapy at the 48 weeks (48-week group). The decision to extend the combination therapy to 72 weeks was made by the patient. To compare the efficacy of the 72- and 48-week courses, all 65 patients of the 72-week group entered this study along with 130 patients of 48-week. The latter group was selected from among the 318 because they matched those patients of the 72-week group with respect to sex, age, and periods from the start of treatment to the initial point of HCV RNA-negative (matched case–control study). The treatment efficacy was evaluated by HCV-RNA positive based on qualitative PCR analysis at the end of treatment (non-virological response; NVR), and by HCV-RNA negative based on qualitative PCR analysis at 24 weeks after the completion of therapy (SVR). Furthermore, LVR was defined as HCV RNA-positive at 12 weeks and negative at 24 weeks after the start of treatment, based on qualitative PCR analysis. All patients fulfilled the following criteria: (1) Negativity for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan), positivity for anti-HCV (third-generation enzyme immunoassay, Chiron Corp., Emerville, CA), and positivity for HCV RNA qualitative analysis with PCR (Amplicor, Roche Diagnostics, Manheim, Germany). (2) Infection with HCV genotype 1b only. (3) A high viral load ($\geq 100 \times 10^3$ IU/ml) by quantitative analysis of HCV RNA with PCR (AMPLICOR GT HCV Monitor v2.0 using the 10-fold dilution method, Roche Molecular Systems Inc., Pleasanton, CA) within the preceding 2 months of enrolment. (4) No hepatocellular carcinoma. (5) Body weight > 40 kg. (6) Lack of coinfection with human immunodeficiency virus. (7) No previous treatment with antiviral or immunosuppressive agents within the preceding 3 months of enrolment. (8) None was an alcoholic; lifetime cumulative alcohol intake was <500 kg. (9) None had other forms of liver diseases, such as hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease, or autoimmune liver disease. (10) None of the females was pregnant or a lactating mother. (11) All patients had completed a 24-week follow-up program after cessation of treatment, and SVR could be evaluated. (12) Each signed a consent form of the study protocol that had been approved by the human ethics review committee. The profile and laboratory data of 195 patients, who entered the matched case–control study, are summarized in Table I.

Laboratory Tests

Blood samples were obtained at least once every month before, during, and after treatment, and were analyzed for alanine aminotransferase (ALT) and HCV-RNA levels. The serum samples were frozen at −80°C within 4 hr of collection and thawed at the time of measurement. HCV genotype was determined by PCR using a mixed primer set derived from the nucleotide sequences of NS5 region [Chayama et al., 1993]. HCV-RNA levels were measured by quantitative PCR (AMPLICOR GT HCV Monitor v2.0 using the 10-fold dilution method, Roche Molecular Systems Inc.) at least once every month before, during, and after therapy. The dynamic range of the assay was 5.0×10^3 to 5.0×10^6 IU/ml. Samples collected during and after therapy that showed undetectable levels of HCV-RNA ($<5.0 \times 10^3$ IU/ml) were also checked by qualitative PCR (AMPLICOR HCV v2.0, Roche Molecular Systems Inc.),