

pared with those in patients with fatty liver due to simple obesity.¹⁵ Recently, we have also shown, using the HCV transgenic mouse model, that the ability of insulin to lower plasma glucose levels is impaired in association with HCV infection,¹⁶ which would be the basis for the frequent development of type 2 diabetes in patients with chronic hepatitis C.^{8,9}

Disturbances in lipid and glucose metabolism are notable features of HCV infection and may be profoundly involved in the pathogenesis of liver diseases. Although the mechanism underlying these phenomena is not yet well understood, the development of clues to correct these metabolic disturbances occurring in HCV infection, which have been recently connected to the poor prognosis of patients with chronic hepatitis C, is awaited. Moreover, a key role for oxidative stress in the pathogenesis of hepatitis C,^{11,12} which may be closely associated with the aforementioned metabolic disorders, has been identified. The association of oxidative stress augmentation in HCV infection with mitochondrial respiratory dysfunction^{10,13,17} suggests that one possibility to ameliorate such a condition is the use of agents that can protect the mitochondrial respiratory function.

We have conducted information retrieval and screening for agents that can protect the mitochondrial respiratory function. Tacrolimus (FK506), which is widely used in organ transplantation, is one such agent with evidence showing protection of the mitochondrial respiratory function,¹⁸⁻²¹ although it shows no antiviral effect. We explored, using transgenic mouse and cultured cell models that express the HCV core protein, whether tacrolimus improves metabolic disturbances including lipid and glucose homeostases as well as oxidative stress augmentation through a possible involvement of mitochondrial function.

Materials and Methods

Transgenic Mouse and Cultured Cells

The production of *HCV core gene* transgenic mice has been described previously.⁶ Mice were cared for according to institutional guidelines with the approval by the institutional review board of the animal care committee, fed an ordinary chow diet (Oriental Yeast Co., Ltd., Tokyo, Japan), and maintained in a specific pathogen-free state. Because there is a sex preference in the development of liver lesions in the transgenic mice, we used only male mice. At least five mice were used in each experiment, and the data were subjected to statistical analysis. HepG2 cell lines expressing the HCV core protein under the control of the CAG promoter (Hep39J, Hep396, and Hep397) or a control HepG2 line (Hepswx) carrying the empty vector were described previously.^{22,23} Bulk HepG2 cells were also used as a control.

Reagents

Cholesterol esters and lipid standards were purchased from Sigma-Aldrich (St. Louis, MO), and glycogen and

amyloglucosidase were obtained from Seikagaku Kogyo (Tokyo, Japan). Other chemicals were of analytical grade and were purchased from Wako Chemicals (Tokyo, Japan). Tacrolimus (FK506) was kindly provided by Astellas Pharma Inc. (Tokyo, Japan). Cyclosporine A (CyA) was purchased from Sigma-Aldrich.

Administration of Tacrolimus and Cyclosporine A

Tacrolimus (0.1 mg/kg b.wt., suspended in mannitol and hydroxychlorinated castor oil [HCO-60]), or vehicle only was administered to the core gene transgenic or control mice i.p., three times per week for 3 months beginning at 3 months of age. For *in vitro* experiments, tacrolimus was added to the culture medium at the final concentration of 0 nmol/L, 10 nmol/L, 100 nmol/L, or 1 μ mol/L. CyA was also added to the culture medium at the same concentrations.

Assessment of Glucose Homeostasis

Blood was drawn at different time points from the tail vein, and plasma glucose concentrations were measured using an automatic biochemical analyzer (DRI-CHEM 3000V, Fuji Film, Tokyo, Japan). The levels of serum insulin were determined by radioimmunoassay (Biotrak, Amersham Pharmacia Biotech, Piscataway, NJ) using rat insulin as a standard. For the determination of the fasting plasma glucose level, the mice were fasted for >16 hours before the study. An insulin tolerance test was performed as described previously.¹⁶

Lipid Extraction, Measurement of Triglyceride Content, and Analysis of Fatty Acid Compositions

Lipid extraction from the mouse liver tissues or cultured cells was performed as described previously.^{15,24} For the analysis of fatty acid compositions, the residue was methylated by the modified Morrison and Smith method with boron trifluoride as a catalyst.²⁵ Fatty acid methyl esters were analyzed using a Shimadzu GC-7A gas chromatograph (Shimadzu Corp., Kyoto, Japan) equipped with a 30-m-long \times 0.3-mm diameter support coated with ethylene glycol succinate.²⁴

Evaluation of Oxidative and Antioxidative System

Lipid peroxidation was estimated spectrophotometrically using thiobarbituric acid-reactive substances and is expressed in terms of malondialdehyde formed per milligram protein. Reduced glutathione and oxidized glutathione levels were measured as described previously.¹⁰ The total amount of glutathione was calculated by adding the amounts obtained for glutathione and oxidized glutathione. For the evaluation of DNA damage in cells, apurinic/apyrimidinic sites were determined using a DNA Damage Quantification Kit (Dojindo Molecular Technolo-

gies, Inc., Tokyo, Japan) following the manufacturer's protocol.

Determination of Reactive Oxygen Species

Cells were plated onto glass coverslips and examined for reactive oxygen species (ROS) production as a marker for oxidative stress. They were loaded for 2 hours with chloromethyl 2',7'-dichlorodihydrofluorescein diacetate (Molecular Probes Inc., Eugene, OR) at a final concentration of 10 $\mu\text{mol/L}$.²⁶ Results were expressed as relative fluorescence intensity and normalized to the control cells. In some experiments, ROS was measured after the incubation with tacrolimus or CyA.

Measurement of Ketone Body Ratio

For the determination of ketone body ratio (KBR), cells were cultured to confluence on a 3.5-cm dish, and the medium was replaced with 700 μl of fresh medium. For arterial KBR, the mice were fasted for >16 hours, followed by the drawing of arterial blood. After a 24-hour incubation, acetoacetate and β -hydroxybutyrate in the medium were measured by monitoring the production or consumption of NADH with a Ketorex kit (Sanwa Chemical, Nagoya, Japan).²⁷ The KBR was calculated as the acetoacetate/ β -hydroxybutyrate ratio.

Microarray Analysis

An Affymetrix GeneChip analysis cDNA array system (Mouse Genome 430A 2.0, Kurabo, Osaka, Japan) was used for the analysis. Two thousand species of mouse DNA fragments were spotted on the filter. Genes that were 1.5-fold increased or decreased in both of the two tacrolimus-treated mice compared with mice treated with vehicle were defined as up-regulated or down-regulated, respectively.

Real-Time PCR and Western Blotting

RNA was prepared from mouse liver tissues using TRIzol LS (Invitrogen, Carlsbad, CA). The first-strand cDNAs were synthesized with a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech, Franklin Lakes, NJ). The fluorescent signal was measured with an ABI Prism 7000 system (Applied Biosystems, Tokyo, Japan).

The genes encoding mouse *tumor necrosis factor (TNF)- α* , *sterol regulatory element binding protein (SREBP)-1c*, *resistin*, *stearoyl-CoA desaturase (SCD)-1*, and *hypoxanthine phosphoribosyltransferase* were amplified with the primer pairs 5'-GACAAGGTGGGCTACGGGCTTG-3' and 5'-TCCCAAATGGGCTCCCTCT-3', 5'-ACGGAGCCATGGATTGCACATTTG-3' and 5'-TACATCTTTAAAGCAGCGGGTGCCGATGGT-3', 5'-GAAGGCACAGCAGTCTTGA-3' and 5'-GCGACCTGCAGCTTACAG-3', 5'-TTCCCTCTGCAAGCTCTAC-3' and 5'-CGCAAGAAGGTGCTAACGAAC-3', and 5'-CCAGCAAGCTTGCAACCTTAACCA-3' and 5'-GTAATGATCAGTCAACGGGGGAC-3', respec-

tively. The sense and antisense primers were located in different exons to avoid false-positive amplification from contaminated genomic DNA. Each PCR product was confirmed as a single band of the correct size by agarose gel electrophoresis (data not shown).

Reporter Assay for SREBP-1c Promoter Activity

A plasmid encoding firefly luciferase under the control of the *SREBP-1c* promoter (pGL3-srebp-1cPro) and a control plasmid encoding *Renilla* luciferase (Promega, Madison, WI) were transfected into 293T cells. Tacrolimus was added at a final concentration of 100 nmol/L to the culture medium of 293T cells transfected with pGL3-srebp-1cPro with or without an expression plasmid of HCV core protein at 24 hours after transfection. Cells were harvested 24 hours after treatment. Luciferase activity was measured by using the dual-luciferase reporter assay system (Promega). Firefly luciferase activity was standardized with that of *Renilla* luciferase, and the results are expressed as the fold-increase in relative luciferase units.

Statistical Analysis

Data are presented as the mean \pm SE. The significance of the difference in means was determined by a Mann-Whitney *U* test wherever appropriate. $P < 0.05$ was considered significant.

Results

Effect of Tacrolimus on Insulin Resistance Induced by HCV

The *core gene* transgenic mice exhibit insulin resistance in the absence of obesity from the age of 2 months.¹⁶ In tacrolimus-treated mice, there was a slight, but not significant, reduction in body weight compared with control mice at the end of tacrolimus administration at 6 months of age (Figure 1A). Tacrolimus administration to the *core gene* transgenic mice restored the plasma glucose levels to within normal limit (Figure 1B) ($P < 0.05$), whereas it caused no significant reduction in the control mice. The plasma glucose levels in the vehicle-treated *core gene* transgenic mice were higher than those in the *core gene* transgenic mice reported previously,¹⁶ probably owing to the older age of mice in the current study than in the previous one. The levels of serum insulin were also significantly reduced by the treatment with tacrolimus for 3 months in the *core gene* transgenic mice, whereas there was no significant change in the control mice (Figure 1C). The reduction in both plasma glucose and serum insulin levels indicates that the administration of tacrolimus restored the resistance to insulin action, which is attributed to the suppression of insulin action in the liver by the *core protein*.¹⁶ Actually, an insulin tolerance test (1 U/kg b.wt.) demonstrated the improvement of insulin action in the tacrolimus-treated *core gene* transgenic mice (Figure 1D).

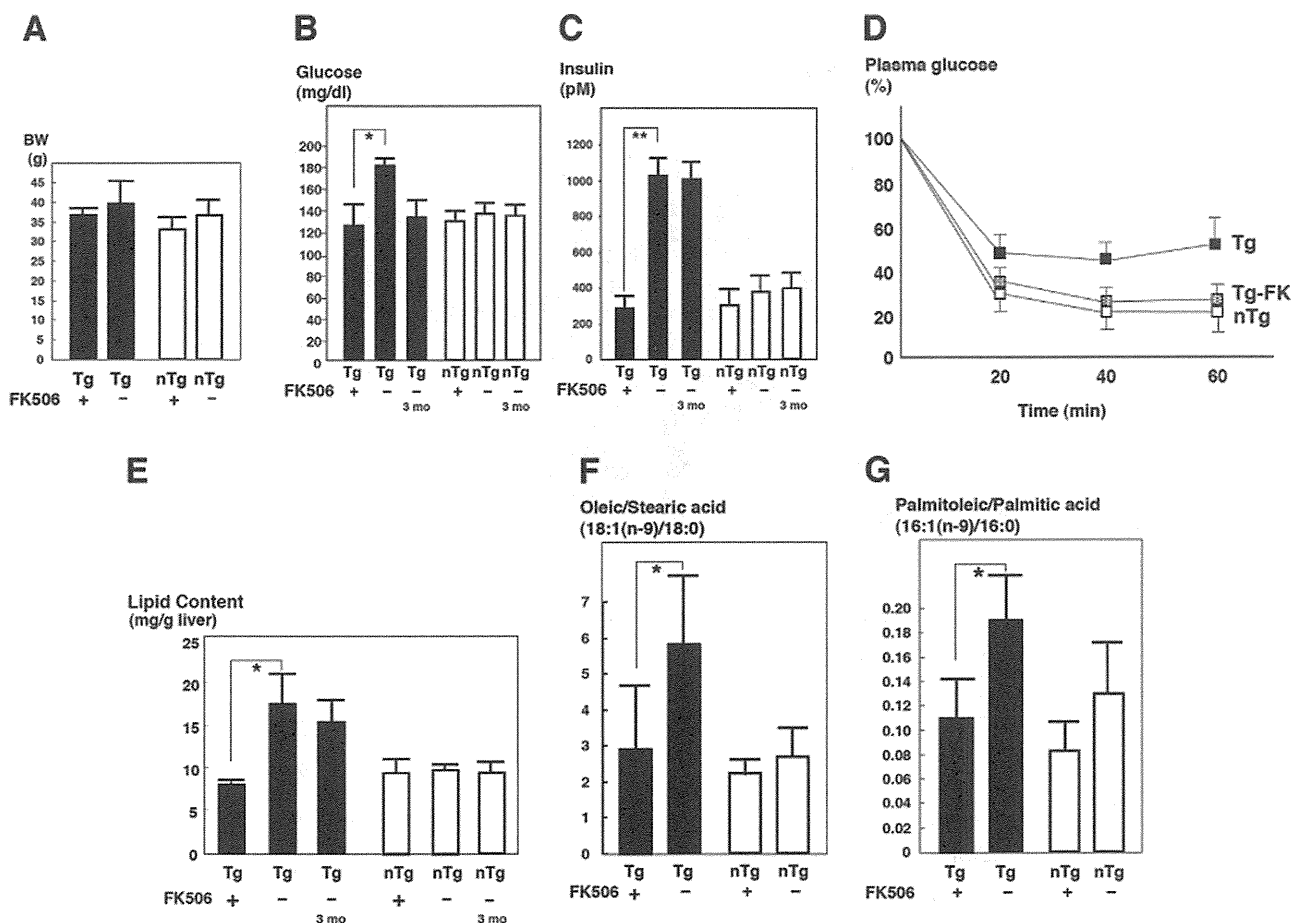


Figure 1. Effect of tacrolimus (FK506) on glucose and lipid metabolism in the core gene transgenic mice. Tacrolimus (0.1 mg/kg b.wt.) or vehicle was administered to core gene transgenic or control mice i.p., three times weekly for 3 months beginning at 3 months of age. **A:** Body weight at the baseline and end of treatment. **B:** Plasma glucose level. **C:** Serum insulin level. **D:** Insulin tolerance test. Black boxes represent core gene transgenic mice; white boxes represent control mice; gray boxes represent core gene transgenic mice treated with tacrolimus (Tg-FK). **E:** Total lipid content in the liver. **F:** Ratio of oleic/stearic acid [18:1(n-9)/18:0]. **G:** Ratio of palmitoleic/palmitic acid [16:1(n-9)/16:0]. black bars represent transgenic mice; white bars represent control mice. Tg 3 mo indicates 3-month-old transgenic mice showing the baseline state just before FK treatment, and Tg indicates 6-month-old transgenic mice, either with or without tacrolimus treatment for 3 months. Values represent the mean \pm SE, $n = 5$ in each group. * $P < 0.05$. Tg, transgenic mice; nTg, nontransgenic control mice. ** $P < 0.01$.

Tacrolimus Improves Lipid Metabolism Disorders in Mice

We then studied whether tacrolimus administration affects lipid metabolism in the mice. The core gene transgenic mice developed a marked hepatic steatosis.^{6,14} In addition, the composition of accumulated lipid was different from that in the fatty liver as a result of simple overnutrition: carbon 18 or 16 monounsaturated fatty acid levels were significantly increased.¹⁵ As shown in Figure 1E, the tacrolimus treatment significantly reduced the lipid content in liver tissues compared with the vehicle treatment of the core gene transgenic mice ($P < 0.05$, $n = 5$ each), whereas there was no change in the control mice. The increased ratios of oleic to stearic acid [18:1(n-9)/18:0] and palmitoleic to palmitic acid [16:1(n-9)/16:0] in the core gene transgenic mice returned to levels similar to those in control mice (Figure 1, F and G) ($P < 0.05$). Thus, the administration of tacrolimus for 3 months restored the abnormalities in lipid metabolism that were induced by the core protein of HCV. Histologically, tacrolimus significantly improved steatosis in the liver of

core gene transgenic mice, in which micro- and macrovesicular lipid droplets were accumulated in hepatocytes, chiefly around the central veins of the liver (Figure 2A). There was no sign of inflammation in the liver with or without the tacrolimus treatment.

Effect of Tacrolimus on Lipid Metabolism in HepG2 Cells Expressing HCV Core Protein

To further prove the ameliorating effect of tacrolimus on lipid metabolism, we then performed experiments using HepG2 cells that express the core protein.^{22,23} HepG2 cells, the lipid metabolism of which is somewhat different from that in normal hepatocytes,²⁸ show a significant increase in the level of 5,8,11-eicosatrienoic acid [20:3(n-9)], as a result of activations of the fatty acid enzymes, Δ^9 -, Δ^6 -, and Δ^5 -desaturases, by the core protein (H. Miyoshi and K. Koike, unpublished data). Incubation of the core-expressing HepG2 cells with tacrolimus at 100 nmol/L and 1 μ mol/L for 48 hours significantly reduced the accumulation of 20:3(n-9), whereas CyA treat-

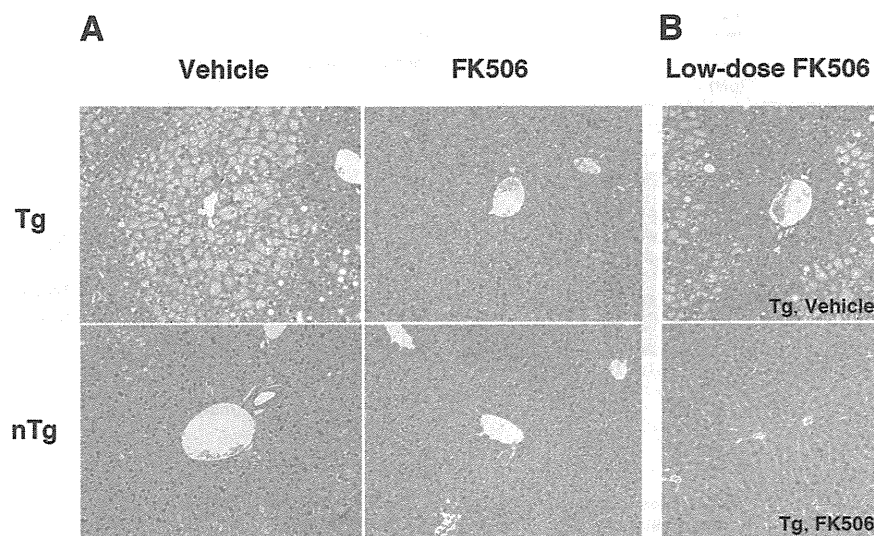


Figure 2. Morphological analysis of the liver of the core gene transgenic mice. Representative cases are shown either treated with tacrolimus (FK506) or vehicle (H&E staining). **A:** There is a prominent improvement of steatosis in the 3-month tacrolimus-treated core gene transgenic mice compared with the vehicle-treated mice. **B:** A prominent improvement in steatosis was also obtained by the administration of one-fifth dose of tacrolimus for 1 month beginning at 3 months of age. For histological analysis, two independent researchers evaluated 40 microscopic fields each, and a representative picture is shown for each category. Original magnification, $\times 125$. Tg, transgenic mice; nTg, nontransgenic control mice.

ment increased the level of 20:3(n-9) in a dose-dependent manner in the core-expressing HepG2 cells (Figure 3, A and B). Neither tacrolimus nor CyA changed the 20:3(n-9) content in HepG2 cells that do not express the core protein.

Low Dose of Tacrolimus Also Ameliorates Steatosis and Insulin Resistance

Because the usual dose of tacrolimus for liver transplantation naturally induces an immunosuppressed state in patients, we conducted a mouse study with a tacrolimus dose lower than that in the aforementioned study. In this low-dose experiment, tacrolimus at 0.02 mg/kg b.wt. (one-fifth of the previous one) was administered to mice for 1 month from the age of 3 months. Similar to the results with the dose of 0.1 mg/kg b.wt., there were significant decreases in the lipid content in the liver (9.5 ± 0.8 [0.02 mg/kg b.wt. tacrolimus] versus 18.7 ± 4.4 [vehicle only] mg/g liver; $P < 0.05$) and serum insulin concentration (96.6 ± 16.9 [0.02 mg/kg b.wt. tacrolimus] versus 1137.1 ± 88.0 [vehicle only] pmol/L; $P < 0.05$) in

the core gene transgenic mice treated with tacrolimus. Histological changes are shown in Figure 2B.

Effect of Tacrolimus on Oxidative Stress and Antioxidative System in Mice

We next examined whether the 3-month administration of tacrolimus affects the redox state in the core gene transgenic mice. In the liver of the core gene transgenic mice, the ROS level was higher than that in the liver of control mice as determined by lipid peroxidation.¹⁰ Treatment with tacrolimus significantly reduced the level of thiobarbituric acid-reactive substances in the liver of the core gene transgenic mice (Figure 4A) ($P < 0.05$). As a result of oxidative stress overproduction, there was damage in the DNA of hepatocytes of the core gene transgenic mice from a young age.¹⁰ To evaluate the effect of tacrolimus on the nuclear DNA damage, the apurinic/aprimidinic site index was determined in liver tissues from the core gene transgenic mice. As shown in Figure 4B, the apurinic/aprimidinic site index in the liver of the core gene transgenic mice, which was significantly higher

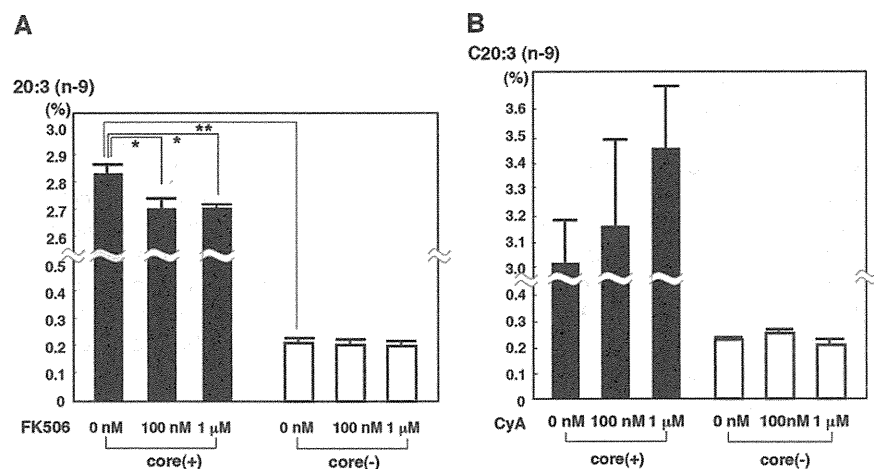


Figure 3. Effect of tacrolimus (FK506) or CyA on fatty acid compositions in HepG2 cells expressing the core protein. The fatty acid compositions of the total cell lipids were analyzed, and the percentage of 5,8,11-eicosatrienoic acid [20:3(n-9)] in the core-expressing and control HepG2 cells was calculated. **A:** Treatment with tacrolimus at 0 nmol/L, 100 nmol/L, or 1 μ mol/L. **B:** Treatment with CyA at 0 nmol/L, 100 nmol/L, or 1 μ mol/L. Black bars represent core-expressing cells; white bars represent control cells. Because similar results were obtained by using Hep39J, Hep396, and Hep397 cell lines, representative results using the Hep39J cell line are shown. Values represent the mean \pm SE; $n = 5$ in each group. * $P < 0.05$ and ** $P < 0.01$.

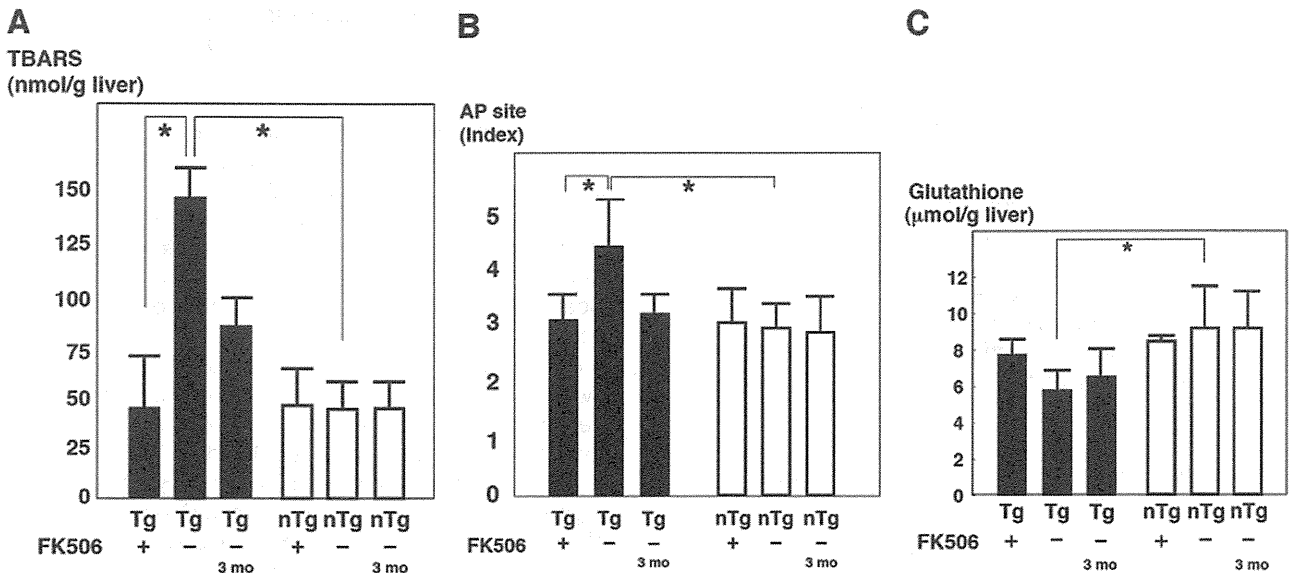


Figure 4. Effect of tacrolimus (FK506) on oxidative stress in the core gene transgenic mice. Tacrolimus (0.1 mg/kg b.wt.) or vehicle only was administered to the core gene transgenic or control mice for 3 months. **A:** Lipid peroxidation in the liver. **B:** apurinic/aprimidinic (AP) site in the liver as a marker of nuclear DNA damage; **C:** Total glutathione level in the liver. Black bars represent transgenic mice; white bars represent control mice. Tg 3 mo indicates 3-month-old transgenic mice, showing the baseline state just before tacrolimus treatment, and Tg indicates 6-month-old transgenic mice, either with or without 3 months of tacrolimus treatment. Values represent the mean \pm SE; $n = 5$ in each group. * $P < 0.05$. Tg, transgenic mice; nTg, nontransgenic control mice. TBARS, thiobarbituric acid-reactive substances.

than that in the control mice, was significantly decreased by the tacrolimus treatment to a level similar to that in the control mice ($P < 0.05$).

The level of glutathione, one of the antioxidant systems, was significantly decreased in the liver of the core gene transgenic mice presumably as a result of oxidative stress overproduction but returned to a level similar to that in the control mice after the 3-month administration of tacrolimus, although the difference was not statistically significant ($P = 0.063$) (Figure 4C). Thus, the oxidative stress augmentation induced by the core protein of HCV was reduced by tacrolimus.

Effect of Tacrolimus on Oxidative Stress in Core-Expressing HepG2 Cells

Evidence for scavenging ROS by the administration of tacrolimus to the mice prompted us to validate this finding using cultured cells. For this purpose, tacrolimus or CyA was added to the culture medium of HepG2 cells that express or do not express the core protein. After 24 hours of incubation, tacrolimus decreased the ROS production level in the core-expressing HepG2 cells in a dose-dependent manner (Figure 5A). In contrast, no decrease but rather an augmentation of ROS production was observed by the treatment with CyA at various concentrations (Figure 5B).

Because dysfunction of the mitochondrial respiratory chain complex 1 is suspected to be the reason for the ROS production associated with HCV infection (H. Miyoshi and K. Koike, unpublished data),^{12,13,17} an increase in the NADH/NAD⁺ ratio, which is caused by the repression of the complex 1 NADH dehydrogenase activity, would be a good marker for the mitochondrial complex 1 dys-

function. Therefore, we evaluated the effect of tacrolimus on the accumulation of NADH in the core-expressing HepG2 cells. The NADH/NAD⁺ ratio, which is strictly estimated from a reciprocal of KBR,^{26,29} was significantly higher in the core gene transgenic mice than in control mice (1/atrial KBR) and in HepG2 cells expressing the core protein than in control cells (1/KBR) (Figure 6A). By the treatment with 1 μ mol/L tacrolimus, the ratio significantly decreased compared with the baseline (Figure 6B), whereas CyA treatment caused no effect in the core-expressing HepG2 cells (Figure 6C), as was the

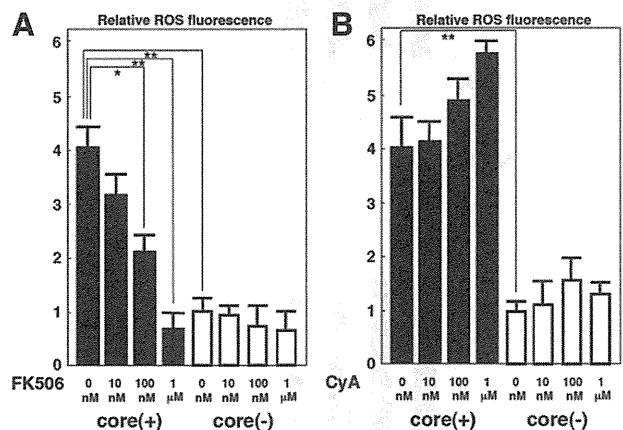


Figure 5. Effect of tacrolimus (FK506) or CyA on ROS production in HepG2 cells expressing the core protein. Results are expressed as relative brightness and normalized to control cells. **A:** Treatment with tacrolimus at 0 nmol/L, 10 nmol/L, 100 nmol/L, or 1 μ mol/L. **B:** Treatment with CyA at 0 nmol/L, 10 nmol/L, 100 nmol/L, or 1 μ mol/L. Black bars represent transgenic mice; white bars represent control cells. Because similar results were obtained by using Hep39J, Hep396, and Hep397 cell lines, representative results using the Hep39J cell line are shown. Values represent the mean \pm SE; $n = 5$ in each group. * $P < 0.05$; ** $P < 0.01$.

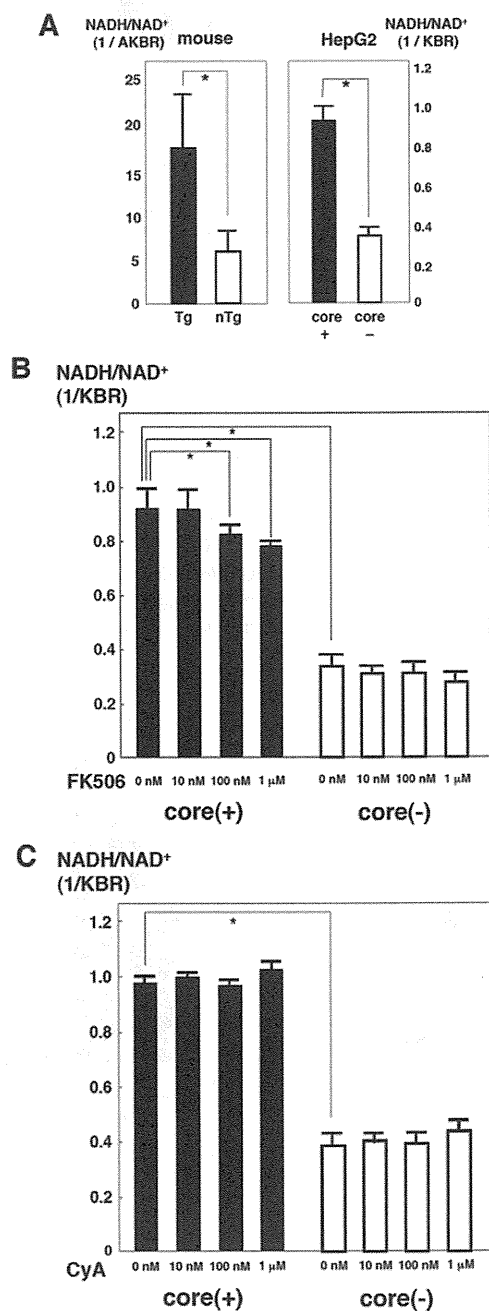
case in the determination of ROS by chloromethyl 2',7'-dichlorodihydrofluorescein diacetate.

Changes in Gene Expression by Tacrolimus Treatment of Mice

We then performed a comprehensive microarray analysis of gene expression in the liver, which was up- or down-regulated by tacrolimus. For this analysis, the tacrolimus-treated mice were compared with the vehicle-treated mice, in two pairs of the core gene transgenic and control mice, respectively. Genes that were 1.5-fold increased or decreased in both of the two tacrolimus-treated mice

compared with those treated with vehicle were defined as up-regulated or down-regulated, respectively. As shown in Table 1, several genes were found to be up-regulated or down-regulated in both the core gene transgenic and control mice after the treatment with tacrolimus for 3 months. A number of genes including that for TNF- α were up- or down-regulated both in the core gene transgenic and control mice. In contrast, the expressions of some genes including that for resistin were differentially regulated between the core gene transgenic and control mice. The expressions of these genes were confirmed by real-time PCR analysis.

Then, to explore the mechanism by which tacrolimus reverses the pathological effect of the core protein in the liver, we examined, by real-time PCR analysis, the expression of some cellular genes including TNF- α , SREBP-1c, SCD-1, and proteasome activator 28- γ . These genes or gene products have been suggested to play a pivotal role in the pathogenesis of HCV-associated liver disease.^{30,31} TNF- α and SREBP-1c genes have been shown to be up-regulated in the liver of the core gene transgenic mice and considered to play a role in the development of insulin resistance and steatosis.^{30,31} By the treatment of the core gene transgenic mice with tacrolimus for 3 months, there was a significant decrease in the mRNA level of both TNF- α and SREBP-1c (Figure 7, A-C) ($P < 0.05$). The SCD-1 mRNA level was also reduced in the tacrolimus-treated core gene transgenic mice. Because down-regulation of SREBP-1c expression by tacrolimus was observed only in the core gene transgenic mice but not in control mice, it is estimated that tacrolimus antagonizes the action of core protein in its transactivating function of the SREBP-1c promoter. The down-regulation of SREBP-1c, then, would lead to the suppression of SCD-1 expression and amelioration of steatosis. We confirmed this by conducting luciferase assays using cultured cells. As shown in Figure 7D, tacrolimus cancelled the effect of the core protein on the activation of SREBP-1c gene promoter. The level of the proteasome activator 28- γ protein, which is indispensable for the action of the core protein in the pathogenesis of HCV-associated liver lesion,³¹ was determined by Western blotting, but there was no change caused by the tacrolimus treatment (data not shown).



Discussion

Antiviral treatment for chronic hepatitis C has advanced markedly. Nearly 50% of patients with chronic hepatitis C

Figure 6. Effect of tacrolimus (FK506) or CyA on NADH accumulation in HepG2 cells expressing the core protein. **A:** NADH/NAD⁺ was determined in mice (left) or HepG2 cells (right) with or without the core protein. **B:** The ketone body ratio was determined in HepG2 cells with or without the core protein after incubation with tacrolimus for 24 hours at 0 nmol/L, 10 nmol/L, 100 nmol/L, or 1 μ mol/L. **C:** The ketone body ratio was determined in HepG2 cells with or without the core protein after incubation with CyA for 24 hours at 0 nmol/L, 10 nmol/L, 100 nmol/L, or 1 μ mol/L. Black bars represent transgenic mice; white bars represent control cells. Because similar results were obtained by using Hep39J, Hep396 and Hep397 cell lines, representative results using the Hep39J cell line are shown. Values represent the mean \pm SE; $n = 5$ in each group. * $P < 0.05$. AKBR, arterial KBR; Tg, transgenic mice; nTg, nontransgenic mice.

Table 1. Genes Whose Expression Levels in the Mouse Liver Were Altered by the Treatment with FK506

	Up-regulated in Tg	Down-regulated in Tg
Up-regulated in nTg	Nuclear factor, erythroid derived 2 DNA segment, human D6S2654E Fatty acid binding protein 5 epidermal squalene epoxidase	Resistin Resistin like alpha Nuclear receptor subfamily 4, group A, member insulin-like growth factor binding protein 1 calcium and integrin binding family member 3
Down-regulated in nTg	Zinc finger protein 69 X-linked lymphocyte-regulated 4 Cytochrome P450, family 2, subfamily b, polypeptide 9 X-linked lymphocyte-regulated 3a Signal sequence receptor, delta	Tumor necrosis factor alpha Cytochrome P450, family 17, subfamily a, polypeptide 1 B-cell leukemia/lymphoma 6

Genes with altered expression in Tg (columns) or in nTg (rows) are described in a 4 × 4 table. Genes that were 1.5-fold increased or decreased in both of the two FK506-treated mice compared with those treated with placebo were defined as up-regulated or down-regulated, respectively. Tg, core gene transgenic mouse; nTg, nontransgenic control mouse.

with HCV genotype 1 and high viral loads achieve a sustained virological response as a result of ribavirin/peginterferon combination therapy.^{32,33} However, the remaining patients who could not achieve sustained virological response continue to experience progression of chronic hepatitis and have a high probability for development of HCC. Although therapies with new agents such as viral protease or RNA polymerase inhibitors are being developed, there is hope for development of the means to retard the progression of chronic hepatitis.

Recently, evidence showing that hepatic steatosis and insulin resistance are crucial determinants of the progression of liver fibrosis has accumulated.^{34–37} Moreover, the importance of oxidative stress, which is closely associated with metabolic disorders such as insulin resistance and steatosis, is implicated in the pathogenesis of HCV-associated liver disease. Given the suggested association of oxidative stress augmentation with the dysfunction of mitochondrial respiration in HCV infection,^{12,13,17} one possibility to ameliorate such a condition is the use of agents that can protect the mitochondrial respiratory function. Tacrolimus is one such agent with evidence of providing protection of the mitochondrial respiratory function,^{18–21} although it does not show an antiviral effect.

In the current study, the administration of tacrolimus significantly improved the disturbances in lipid and glucose metabolism both *in vivo* and *in vitro*. As disorders of

lipid metabolism associated with HCV infection, hepatic steatosis and increases in monounsaturated fatty acid levels have been demonstrated.^{3,4,6,7,15} The latter is caused by the activation of fatty acid enzymes such as Δ^9 - or Δ^6 -desaturase, resulting in increases in 18:1(n-9)/18:0 and 16:1(n-9)/16:0 ratios (H. Miyoshi and K. Koike, unpublished data).¹⁵ Tacrolimus ameliorated these lipid alterations associated with HCV infection with no impact on mouse body weight. Tacrolimus also improved the insulin resistance in the HCV mouse model, in which tyrosine phosphorylation of insulin receptor substrate-1 is impaired by the HCV core protein.¹⁶

Moreover, tacrolimus treatment ameliorated oxidative stress augmentation, which is considered to play a pivotal role in the progression of liver disease or the development of HCC in HCV infection.^{10–13} In mice transgenic for the HCV core gene, in which DNA damage develops because of oxidative stress augmentation,¹³ tacrolimus decreased the levels of peroxylipid and DNA damage formations. Dysfunction of the mitochondrial respiratory chain complex 1 is suspected to be a source of ROS overproduction in HCV infection.^{12,13,17} To assess changes in mitochondrial complex 1 function caused by tacrolimus, the NADH/NAD⁺ ratio, which reflects the complex 1 NADH dehydrogenase activity, was determined in HepG2 cells expressing the core protein. The NADH/NAD⁺ ratio, which is strictly estimated from a reciprocal of KBR (1/atrial KBR),^{26,29} was significantly re-

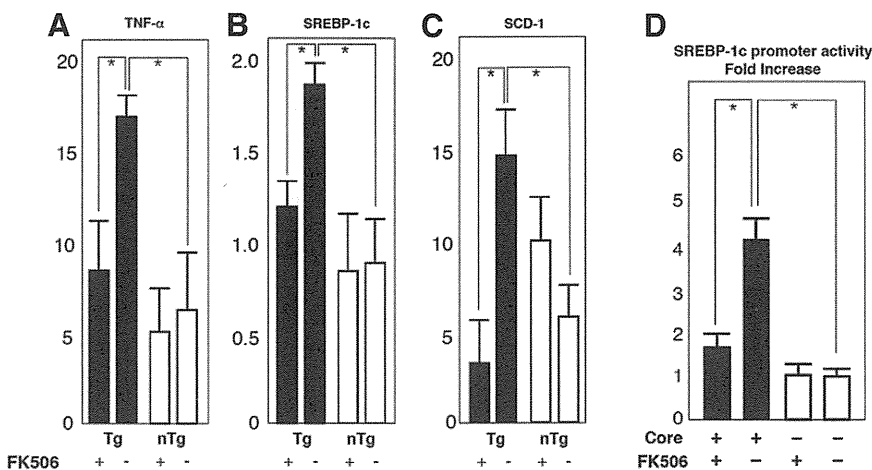


Figure 7. A–C: Effect of tacrolimus (FK506) on mRNA levels of cellular genes. The mRNA levels of TNF- α (A), SREBP-1c (B), and SCD-1 (C) genes were determined by real-time PCR analysis in the tacrolimus- or vehicle-treated mouse livers. The transcriptions of the genes were normalized with that of hypoxanthine phosphoribosyltransferase, and the values are expressed as relative activities. D: Effect of tacrolimus on the transactivating function of the core protein on the SREBP-1c promoter. A luciferase assay was performed using a plasmid encoding firefly luciferase under the control of the SREBP-1c promoter with or without the expression of HCV core protein. Tacrolimus was added at a final concentration of 100 nmol/L to the culture medium. Black bars represent transgenic mice; white bars represent control cells. Values represent the mean \pm SE; $n = 5$ in each group. * $P < 0.05$. Tg, transgenic mice; nTg, nontransgenic mice.

duced by the addition of tacrolimus but not CyA. Thus, tacrolimus protected the mitochondrial respiratory chain complex 1 function from the impact of the core protein, decreased oxidative stress, and improved steatosis and insulin resistance.

Some of features induced by the core protein including steatosis, insulin, and DNA damage were already present in the core gene transgenic mice at 3 months of age as the baseline, and those were improved by tacrolimus treatment. This fact indicates that tacrolimus is not only preventing the development of core-induced features but also reversing such changes in the mouse liver.

The tacrolimus dose used in the current study was 0.1 mg/kg b.wt. This is the same dose as that used in recipients of liver or kidney transplantation. The result of a subexperiment with a lower tacrolimus dose of 0.02 mg/kg b.wt. was similar to that with the dose of 0.1 mg/kg b.wt. This finding is promising because it indicates that the "anti-core protein effect" may be achievable at such a low dose of tacrolimus without provoking strong immunosuppression. The tacrolimus concentration (100 nmol/L) that caused the anti-core protein effect in the cultured cell study is similar to that in the blood of recipients of liver transplantation and much lower than those used in previous studies.^{19,38} In the current study, tacrolimus was administered only i.p., although it tacrolimus is administered i.v. or p.o. in humans. Therefore, a concern may arise regarding the administration route. Because the bioavailability of tacrolimus is approximately 25% (range from 5 to 93%) in human patients,³⁹ a difference in the concentrations of tacrolimus may be possible between i.p. and p.o. administration. However, in human patients, target levels of tacrolimus concentration are generally achieved by p.o. administration as the maintenance therapy. Therefore, the target concentration would be achieved in mouse models by p.o. administration for 3 months as it is in human patients. Our current results strongly support the notion that tacrolimus can protect the mitochondrial respiratory function, resulting in a reduction of ROS production.

There is also a controversy concerning the effect of tacrolimus on glucose homeostasis. Post-transplantation diabetes is a complication in kidney or liver transplantation.^{40,41} *In vivo* and *in vitro* studies have shown that tacrolimus may inhibit insulin secretion from the pancreatic β -cells.⁴⁰ Thus, tacrolimus may have a potential to induce diabetes. However, there have been no well designed studies on this specific point: in one study, corticosteroid withdrawal from tacrolimus-based immunosuppression reduced insulin resistance without changing insulin secretion.⁴¹ In our study using the HCV mouse model, tacrolimus administration at the dose similar to those in organ transplant recipients decreased serum insulin levels without increasing plasma glucose levels. These results point toward the future use of tacrolimus *in vivo* for the amendment of metabolic abnormalities, such as steatosis and insulin resistance, associated with HCV infection. However, it should be noted that there is a difference between our mouse model and human patients. Organ transplant recipients generally have injury to other bodily organs after a prolonged course of illness,

whereas the mouse model we have exploited does not. In addition, our mouse model originally has insulin resistance with the presence of hyperplasia of Langerhans islands.¹⁶ Therefore, the effect of tacrolimus on glucose homeostasis in the current mouse study may not be exactly applicable to human patients.

The results of the gene expression analysis by microarray and subsequent real-time PCR were of considerable interest. Tacrolimus reduced the mRNA levels of TNF- α , SCD-1, and SREBP-1c genes, which are elevated in both patients with chronic hepatitis C and HCV core gene transgenic mice.^{30,31} The elevation in the TNF- α level causes insulin resistance *in vivo*, which is also observed in HCV core gene transgenic mice.¹⁶ The elevations in SREBP-1c and SCD-1 gene mRNA levels cause the overproduction of triglycerides, leading to the development of steatosis. The reductions in the expression levels of these genes may explain the effect of tacrolimus on the improvement of steatosis, insulin resistance, and oxidative stress in these HCV models. Although recent investigations have shown that the immunosuppressive drugs tacrolimus and rapamycin inhibit the expression of different inflammatory mediators,^{42,43} the anti-inflammatory functions of these drugs are not well established. Our *in vitro* and *in vivo* experiments confirmed that tacrolimus inhibited the induction of ROS generation, which is mediated by the core protein. Our data indicate that the inhibition of ROS formation may explain part of the favorable effect of immunosuppressive agents on inflammatory conditions.

In conclusion, our results demonstrate that tacrolimus has protective potential against damage caused by the HCV core protein including the induction of steatosis, insulin resistance, and oxidative stress, both in mice and cultured cells. Although more studies are required to elucidate the precise mechanism underlying the potential of tacrolimus in reversing the pathogenesis in HCV infection, these results may provide new therapeutic tools for chronic hepatitis C, in which oxidative stress and abnormalities in lipid and glucose metabolism contribute to liver pathogenesis.

References

1. Saito I, Miyamura T, Ohbayashi A, Harada H, Katayama T, Kikuchi S, Watanabe Y, Koi S, Onji M, Ohta Y, Choo Q, Houghton M, Kuo G: Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc Natl Acad Sci USA* 1990, 87:6547-6549
2. Simonetti RG, Camma C, Fiorello F, Cottone M, Rapicetta M, Marino L, Fiorentino G, Craxi A, Ciccaglione A, Giuseppetti R, Stroffolini T, Pagliaro L: Hepatitis C virus infection as a risk factor for hepatocellular carcinoma in patients with cirrhosis. *Ann Intern Med* 1992, 116:97-102
3. Scheuer PJ, Ashrafzadeh P, Sherlock S, Brown D, Dusheiko GM: The pathology of chronic hepatitis C. *Hepatology* 1992, 15:567-571
4. Bach N, Thung SN, Schaffner F: The histological features of chronic hepatitis C and autoimmune chronic hepatitis: a comparative analysis. *Hepatology* 1992, 15:572-577
5. Barba G, Harper F, Harada T, Kohara M, Goulinet S, Matsuura Y, Eder G, Schaff Z, Chapman MJ, Miyamura T, Bréchet C: Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. *Proc Natl Acad Sci USA* 1997, 94:1200-1205
6. Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Ishibashi K, Matsuura Y, Miyamura T, Koike K: Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. *J Gen Virol* 1997, 78:1527-1531

7. Lerat H, Honda M, Beard MR, Loesch K, Sun J, Yang Y, Okuda M, Gosert R, Xiao SY, Weinman SA, Lemon SM: Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. *Gastroenterology* 2002, 122:352–365
8. Caronia S, Taylor K, Pagliaro L, Carr C, Palazzo U, Petrik J, O'Rahilly S, Shore S, Tom BD, Alexander GJ: Further evidence for an association between non-insulin-dependent diabetes mellitus and chronic hepatitis C virus infection. *Hepatology* 1999, 30:1059–1063
9. Mehta SH, Brancati FL, Sulkowski MS, Strathdee SA, Szklo M, Thomas DL: Prevalence of type 2 diabetes mellitus among persons with hepatitis C virus infection in the United States. *Ann Intern Med* 2000, 133:592–599
10. Choi J, Ou JH: Mechanisms of liver injury. III. Oxidative stress in the pathogenesis of hepatitis C virus. *Am J Physiol Gastrointest Liver Physiol* 2006, 290:G847–G851
11. Koike K, Miyoshi H: Oxidative stress and hepatitis C viral infection. *Hepatol Res* 2006, 34:65–76
12. Korenaga M, Wang T, Li Y, Showalter LA, Chan T, Sun J, Weinman SA: Hepatitis C virus core protein inhibits mitochondrial electron transport and increases reactive oxygen species (ROS) production. *J Biol Chem* 2005, 280:37481–37488
13. Moriya K, Nakagawa K, Santa T, Shintani Y, Fujie H, Miyoshi H, Tsutsumi T, Miyazawa T, Ishibashi K, Horie T, Imai K, Miyamura T, Kimura S, Koike K: Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocarcinogenesis. *Cancer Res* 2001, 61:4365–4370
14. Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Matsuura Y, Kimura S, Miyamura T, Koike K: The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat Med* 1998, 4:1065–1067
15. Moriya K, Todoroki T, Tsutsumi T, Yotsuyanagi H, Tsutsumi T, Ishibashi K, Takayama T, Makuuchi M, Watanabe K, Miyamura T, Kimura S, Koike K: Increase in the concentration of carbon 18 monounsaturated fatty acids in the liver with hepatitis C: analysis in transgenic mice and humans. *Biophys Biochem Res Commun* 2001, 281:1207–1212
16. Shintani Y, Fujie H, Miyoshi H, Tsutsumi T, Kimura S, Moriya K, Koike K: Hepatitis C virus and diabetes: direct involvement of the virus in the development of insulin resistance. *Gastroenterology* 2004, 126:840–848
17. Piccoli C, Scrima R, Quarato G, D'Aprile A, Ripoli M, Lecce L, Boffoli D, Moradpour D, Capitanio N: Hepatitis C virus protein expression causes calcium-mediated mitochondrial bioenergetic dysfunction and nitro-oxidative stress. *Hepatology* 2007, 46:58–65
18. Cetinkale O, Konukoğlu D, Senel O, Kemerli GD, Yazar S: Modulating the functions of neutrophils and lipid peroxidation by FK506 in a rat model of thermal injury. *Burns* 1999, 25:105–112
19. Kaibori M, Inoue T, Tu W, Oda M, Kwon AH, Kamiyama Y, Okumura T: FK506, but not cyclosporin A, prevents mitochondrial dysfunction during hypoxia in rat hepatocytes. *Life Sci* 2001, 69:17–26
20. Keswani SC, Chander B, Hasan C, Griffin JW, McArthur JC, Hoke A: FK506 is neuroprotective in a model of antiretroviral toxic neuropathy. *Ann Neurol* 2003, 53:57–64
21. Kaymaz M, Emmez H, Bukan N, Dursun A, Kurt G, Paçsaoğlu H, Paçsaoğlu A: Effectiveness of FK506 on lipid peroxidation in the spinal cord following experimental traumatic injury. *Spinal Cord* 2005, 43:22–26
22. Ruggieri A, Murdolo M, Harada T, Miyamura T, Rapicetta M: Cell cycle perturbation in a human hepatoblastoma cell line constitutively expressing hepatitis C virus core protein. *Arch Virol* 2004, 149:61–74
23. Aizaki H, Harada T, Otsuka M, Seki N, Matsuda M, Li YW, Kawakami H, Matsuura Y, Miyamura T, Suzuki T: Expression profiling of liver cell lines expressing entire or parts of hepatitis C virus open reading frame. *Hepatology* 2002, 36:1431–1438
24. Todoroki T, Imai K, Matsumoto K, Kano S: Initial deactivation of Florisil adsorbent for column chromatographic separation of lipids. *Analyst* 1983, 108:1267–1269
25. Morrison WR, Smith LM: Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. *J Lipid Res* 1964, 5:600–608
26. Gelasco AK, Raymond JR: Indoxyl sulfate induces complex redox alterations in mesangial cells. *Am J Physiol Renal Physiol* 2006, 290:F1551–F1558
27. Williamson DH, Mellanby J, Krebs HA: Enzymic determination of α - β -hydroxybutyric acid and acetoacetic acid in blood. *Biochem J* 1962, 82:90–96
28. Choi Y, Park Y, Pariza MW, Ntambi JM: Regulation of stearoyl-CoA desaturase activity by the *trans*-10,*cis*-12 isomer of conjugated linoleic acid in HepG2 cells. *Biochem Biophys Res Commun* 2001, 284:689–693
29. Williamson DH, Lund P, Krebs HA: The redox state of free nicotinamide-adenine dinucleotide in the cytoplasm and mitochondria of rat liver. *Biochem J* 1967, 103:514–527
30. Tsutsumi T, Suzuki T, Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Matsuura Y, Kimura S, Koike K, Miyamura T: Intrahepatic cytokine expression and AP-1 activation in mice transgenic for hepatitis C virus core protein. *Virology* 2002, 304:415–424
31. Moriishi K, Mochizuki R, Moriya K, Miyamoto H, Mori Y, Abe T, Murata S, Tanaka K, Suzuki T, Miyamura T, Koike K, Matsuura Y: Critical role of PA28 γ in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis. *Proc Natl Acad Sci USA* 2007, 104:1661–1666
32. Hoofnagle JH, Seeff LB: Peginterferon and ribavirin for chronic hepatitis C. *N Engl J Med* 2006, 355:2444–2451
33. Koike K: Antiviral treatment of hepatitis C: present status and future prospects. *J Infect Chemother* 2006, 12:227–232
34. Adinolfi LE, Gambardella M, Andreana A, Tripodi MF, Utili R, Ruggiero G: Steatosis accelerates the progression of liver damage of chronic hepatitis C patients and correlates with specific HCV genotype and visceral obesity. *Hepatology* 2001, 33:1358–1364
35. Patton HM, Patel K, Behling C, Tripodi MF, Utili R, Ruggiero G: The impact of steatosis on disease progression and early and sustained treatment response in chronic hepatitis C patients. *J Hepatol* 2004, 40:484–490
36. Hui JM, Sud A, Farrell GC, Bandara P, Byth K, Kench JG, McCaughan GW, George J: Insulin resistance is associated with chronic hepatitis C virus infection and fibrosis progression. *Gastroenterology* 2003, 125:1695–1704
37. Hickman IJ, Powell EE, Prins JB, Clouston AD, Ash S, Purdie DM, Jonsson JR: Insulin resistance is associated with chronic hepatitis C and virus infection fibrosis progression. *J Hepatol* 2003, 39:1042–1048
38. Han SY, Chang EJ, Choi HJ, Kwak CS, Suh SI, Bae JH, Park SB, Kim HC, Mun KC: Effect of tacrolimus on the production of oxygen free radicals in hepatic mitochondria. *Transplant Proc* 2006, 38:2242–2243
39. Staatz CE, Tett SE: Clinical pharmacokinetics and pharmacodynamics of tacrolimus in solid organ transplantation. *Clin Pharmacokinet* 2004, 43:623–653
40. Penforis A, Kury-Paulin S: Immunosuppressive drug-induced diabetes. *Diabetes Metab* 2006, 32:539–546
41. van Hooff JP, Christiaans MH, van Duijnhoven EM: Evaluating mechanisms of post-transplant diabetes mellitus: *Nephrol Dial Transplant* 2004, 19(Suppl 6):vi8–vi12
42. Vigil SV, de Liz R, Medeiros YS, Fröde TS: Efficacy of tacrolimus in inhibiting inflammation caused by carrageenan in a murine model of air pouch. *Transpl Immunol* 2008, 19:25–29
43. Pereira R, Medeiros YS, Fröde TS: Antiinflammatory effects of tacrolimus in a mouse model of pleurisy. *Transpl Immunol* 2006, 16:105–111

Dysfunction of Autophagy Participates in Vacuole Formation and Cell Death in Cells Replicating Hepatitis C Virus^{∇§}

Shuhei Taguwa,^{1†} Hiroto Kambara,^{1†} Naonobu Fujita,² Takeshi Noda,² Tamotsu Yoshimori,² Kazuhiko Koike,³ Kohji Moriishi,⁴ and Yoshiharu Matsuura^{1*}

Department of Molecular Virology, Research Institute for Microbial Diseases,¹ and Department of Genetics, Graduate School of Medicine,² Osaka University, Osaka 565-0871, Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, Tokyo 113-8655,³ and Department of Microbiology, Faculty of Medicine, Yamanashi University, Yamanashi 409-3898,⁴ Japan

Received 22 August 2011/Accepted 4 October 2011

Hepatitis C virus (HCV) is a major cause of chronic liver diseases. A high risk of chronicity is the major concern of HCV infection, since chronic HCV infection often leads to liver cirrhosis and hepatocellular carcinoma. Infection with the HCV genotype 1 in particular is considered a clinical risk factor for the development of hepatocellular carcinoma, although the molecular mechanisms of the pathogenesis are largely unknown. Autophagy is involved in the degradation of cellular organelles and the elimination of invasive microorganisms. In addition, disruption of autophagy often leads to several protein deposition diseases. Although recent reports suggest that HCV exploits the autophagy pathway for viral propagation, the biological significance of the autophagy to the life cycle of HCV is still uncertain. Here, we show that replication of HCV RNA induces autophagy to inhibit cell death. Cells harboring an HCV replicon RNA of genotype 1b strain Con1 but not of genotype 2a strain JFH1 exhibited an incomplete acidification of the autolysosome due to a lysosomal defect, leading to the enhanced secretion of immature cathepsin B. The suppression of autophagy in the Con1 HCV replicon cells induced severe cytoplasmic vacuolation and cell death. These results suggest that HCV harnesses autophagy to circumvent the harmful vacuole formation and to maintain a persistent infection. These findings reveal a unique survival strategy of HCV and provide new insights into the genotype-specific pathogenicity of HCV.

Hepatitis C virus (HCV) is a major causative agent of blood-borne hepatitis and currently infects at least 180 million people worldwide (58). The majority of individuals infected with HCV develop chronic hepatitis, which eventually leads to liver cirrhosis and hepatocellular carcinoma (25, 48). In addition, HCV infection is known to induce extrahepatic diseases such as type 2 diabetes and malignant lymphoma (20). It is believed that the frequency of development of these diseases varies among viral genotypes (14, 51). However, the precise mechanism of the genotype-dependent outcome of HCV-related diseases has not yet been elucidated. Despite HCV's status as a major public health problem, the current therapy with pegylated interferon and ribavirin is effective in only around 50% of patients with genotype 1, which is the most common genotype worldwide, and no effective vaccines for HCV are available (35, 52). Although recently approved protease inhibitors for HCV exhibited a potent antiviral efficacy in patients with genotype 1 (36, 43), the emergence of drug-resistant mutants is a growing problem (16). Therefore, it is important to clarify the life cycle and pathogenesis of HCV for the development of more potent remedies for chronic hepatitis C.

HCV belongs to the genus *Hepacivirus* of the family *Flaviviridae* and possesses a single positive-stranded RNA genome with a nucleotide length of 9.6 kb, which encodes a single polyprotein consisting of approximately 3,000 amino acids (40). The precursor polyprotein is processed by host and viral proteases into structural and nonstructural (NS) proteins (34). Not only viral proteins but also several host factors are required for efficient replication of the HCV genome, where NS5A is known to recruit various host proteins and to form replication complexes with other NS proteins (39). In the HCV-propagating cell, host intracellular membranes are reconstructed for the viral niche known as the membranous web, where it is thought that progeny viral RNA and proteins are concentrated for efficient replication and are protected from defensive degradation, as are the host protease and nucleases (38).

Autophagy is a bulk degradation process, wherein portions of cytoplasm and organelles are enclosed by a unique membrane structure called an autophagosome, which subsequently fuses with the lysosome for degradation (37, 60). Autophagy occurs not only in order to recycle amino acids during starvation but also to clear away deteriorated proteins or organelles irrespective of nutritional stress. In fact, the deficiency of autophagy leads to the accumulation of disordered proteins that can ultimately cause a diverse range of diseases, including neurodegeneration and liver injury (12, 29, 30), and often to type 2 diabetes and malignant lymphoma (9, 32).

Recently, it has been shown that autophagy is provoked upon replication of several RNA viruses and is closely related to their propagation and/or pathogenesis. Cocksackievirus B3

* Corresponding author. Mailing address: Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, 3-1, Yamadaoka, Suita-shi, Osaka 565-0871, Japan. Phone: 81-6-6879-8340. Fax: 81-6-6879-8269. E-mail: matsuura@biken.osaka-u.ac.jp.

† These authors contributed equally to this work.

§ Supplemental material for this article may be found at <http://jvi.asm.org/>.

∇ Published ahead of print on 12 October 2011.

utilizes autophagic membrane as a site of genome replication, whereas influenza virus attenuates apoptosis through the induction of autophagy (10, 59). Moreover, several groups have reported that HCV induces autophagy for infection or replication (5, 49); however, the role(s) of autophagy in the propagation of HCV is still controversial and the involvement of autophagy in the pathogenesis of HCV has not yet been clarified. In this study, we examined the biological significance of the autophagy observed in cells in which the HCV genome replicates.

MATERIALS AND METHODS

Plasmids. The plasmids pmStrawberry-C1, pmStrawberry-Atg4B^{C74A}, pmRFP-GFP-LC3, pEGFP-LC3, and pEGFP-Atg16L were described previously (7, 8, 24). The plasmids pFGR-JFH1 and pSGR-JFH1 were kind gifts from T. Wakita.

Cell culture. All cell lines were cultured at 37°C under a humidified atmosphere with 5% CO₂. Huh7 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), nonessential amino acids, 100 U/ml penicillin, and 100 mg/ml streptomycin. For the starvation, the cells were cultivated with Earle's balanced salt solution (EBSS) (Sigma) for 6 h. HCV replicon cells were established as described previously (53). The plasmid pairs pFK-I₃₈₉ neo/NS3-3'/NK5.1 and pFK-I₃₈₉ neo/FGR/NK5.1 and pFGR-JFH1 and pSGR-JFH1 were linearized with *Scal* or *Xba*I. The plasmids pFGR-JFH1 and pSGR-JFH1 were treated with mung bean exonuclease. The linearized DNA was transcribed *in vitro* by using the MEGAscript T7 kit (Applied Biosystems) according to the manufacturer's protocol. The transcribed RNA was electroporated into cells under conditions of 270 V and 960 mF using a Gene Pulser (Bio-Rad). All HCV replicon cells were maintained in DMEM containing 10% FBS, nonessential amino acids, and 1 mg/ml G418 (Nacalai).

Reagents and antibodies. Concanamycin A and baflomycin A1 were purchased from Sigma and Fluka, respectively. E64D and pepstatin A were from Peptide Institute Inc. Rabbit anti-HCV NS5A polyclonal antibody was described previously (45). Mouse monoclonal anti-JEV NS3 antibody was prepared by immunization using the recombinant protein spanning amino acid residues 171 to 619 of JEV NS3. Rabbit polyclonal anti-LC3 (PM036), mouse monoclonal anti-RFP (8D6), and anti-62/SQSTM1 (5F2) antibodies were purchased from Medical & Biological Laboratories. Rabbit polyclonal anti-cathepsin B (FL-339) and mouse monoclonal anti-LAMP1 (H4A3) antibodies were from Santa Cruz Biotechnology. Mouse monoclonal anti-HCV NS5A (HCM-131-5), rabbit polyclonal anti-β-actin, and mouse monoclonal anti-Golgin97 (CDF4) antibodies were from Austral Biologicals, Sigma, and Invitrogen, respectively. Mouse monoclonal and rabbit polyclonal anti-cathepsin B antibodies were from Calbiochem. Mouse monoclonal anti-p62/SQSTM1 (5F2) and anti-ATP6V0D1 (ab56441) antibodies were from Abcam. Rabbit polyclonal anti-Atg4B antibody was from Sigma. Mouse anti-double-stranded RNA (dsRNA) IgG2a (J2 and K1) antibodies were from Biocenter Ltd. (Szirak, Hungary).

Transfection, infection, and immunoblotting. Transfection and infection were carried out as described previously (53). Each lysosome-enriched fraction was isolated by using the Lysosome Enrichment Kit for Tissue and Cultured Cells (Pierce) according to the manufacturer's protocol. Samples were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes (Millipore) and were reacted with the appropriate antibodies. The immune complexes were visualized with Super Signal West Femto substrate (Pierce) and detected by an LAS-3000 image analyzer system (Fujifilm). The protein bands of LC3 and β-actin were quantified by Multi Gauge software (Fujifilm), and the values of LC3 were normalized to those of β-actin.

Fluorescence microscopy. Cells were cultured on glass slides and then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 30 min. After being washed twice with PBS, the cells were permeabilized at room temperature for 20 min with PBS containing 0.25% saponin and then blocked with PBS containing 0.2% gelatin (gelatin-PBS) for 60 min at room temperature. The cells were incubated with gelatin-PBS containing appropriate antibodies at 37°C for 60 min and washed three times with PBS containing 1% Tween 20 (PBST). The resulting cells were incubated with gelatin-PBS containing corresponding fluorescent-conjugated secondary antibodies at 37°C for 60 min and then washed three times with PBST. The stained cells were covered with Vectashield mounting medium containing DAPI (4',6-diamidino-2-phenylin-

dole) (Vector Laboratories Inc.) and observed with a FluoView FV1000 laser scanning confocal microscope (Olympus). Time-lapse video microscopy was performed at 37°C with a DeltaVision microscope system (Applied Precision Inc.) equipped with a ΔTC3 culture dish system (Biopetechs) for temperature control.

Quantification of pro-cathepsin B. Each cell line was seeded on 12-well type I collagen-coated dishes (IWAKI) and cultured for 48 h. The supernatant and the cells were harvested and subjected to quantification of pro-cathepsin B by using Quantikine human pro-cathepsin B immunoassay (R&D Systems) according to the manufacturer's protocol.

Statistical analysis. Estimated values were represented as the means ± standard deviations. The significance of differences in the means was determined by Student's *t* test.

RESULTS

Autophagy is induced in the HCV replicating cell in a strain-dependent manner. To determine whether autophagy is induced during the replication of HCV, we investigated the phosphoethanolamine (PE) conjugation of LC3 in HCV replicon cells in which HCV RNA was autonomously replicating. As shown in Fig. 1A, the amounts of PE-conjugated LC-3 (LC3-II), a conventional marker for an autophagosomal membrane, in Huh7 cells were slightly increased by starvation, in conjunction with a reduction of the unmodified LC-3 (LC3-I). In contrast, the amount of LC3-II was significantly increased in the subgenomic and full genomic HCV replicon cells of the genotype 1b strain Con1 (SGR^{Con1} and FGR^{Con1}), whereas a small amount of LC3-II was detected in the full genomic replicon cells of the genotype 2a strain JFH1 (FGR^{JFH1}). We also examined the subcellular localization of LC3 by using confocal microscopy. Although LC3 was diffusely detected in the cytoplasm of naïve Huh7 cells, small foci of the accumulated LC3 appeared after starvation (Fig. 1B), whereas many LC3 foci that were larger in size than those in the starved cells appeared in the cytoplasm, particularly near the nucleus, in both SGR^{Con1} and FGR^{Con1} cells. However, a low level of LC3 focus formation comparable to that in the starved cells was observed in the FGR^{JFH1} cells. Most of the LC3 foci were not colocalized with NS5A, an HCV protein of the viral replication complex, in the HCV replicon cells, as reported previously (49). Elimination of HCV RNA from the SGR^{Con1} cells by treatment with alpha interferon (SGR^{Con1} abrogated the lipidation and accumulation of LC3 (Fig. 1C and D). Interestingly, overexpression of the HCV polyprotein of genotype 1b by an expression plasmid induced no autophagy (data not shown), suggesting that replication of viral RNA is required for induction of autophagy. Furthermore, neither lipidation nor accumulation of LC3 was observed in SGR^{JEV} cells harboring subgenomic replicon RNA cells of Japanese encephalitis virus (JEV), which is also a member of the family *Flaviviridae* (Fig. 1C and D). These results suggest that replication of HCV but not that of JEV induces autophagy.

The autophagy flux is impaired in the replicon cells of HCV strain Con1 after a step of autophagosome formation. To further examine the autophagy induced in the HCV replicon cells in more detail, Huh7 and SGR^{Con1} cells were treated with pepstatin A and E64D, inhibitors of aspartic protease and cysteine protease, respectively. In this assay, treatment of intact cells capable of inducing autophagy with the inhibitors increases the amount of LC3-II, whereas no increase is observed in cells impaired in the autophagic degradation. The amount of LC3-II was significantly increased in the naïve Huh7

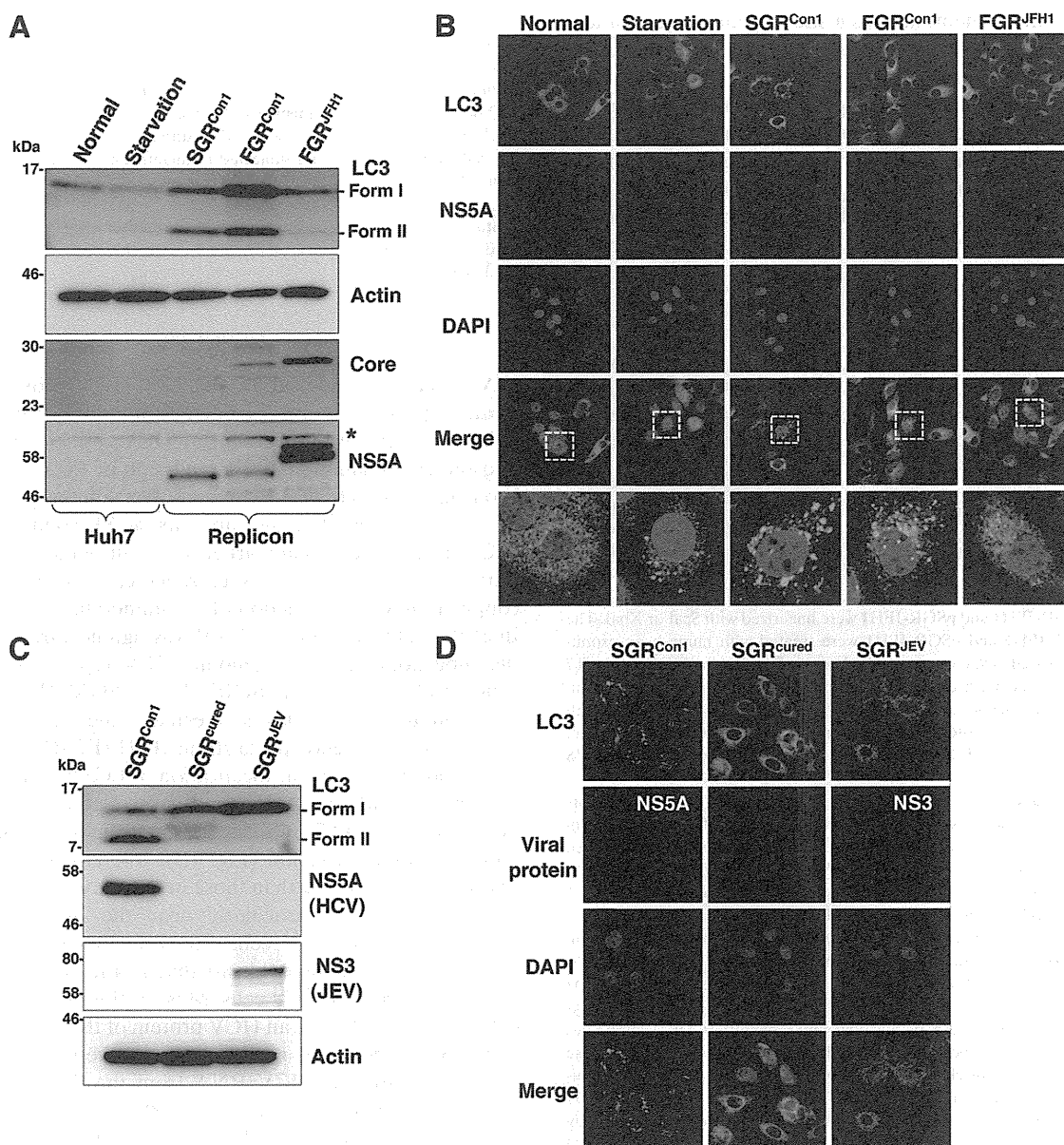


FIG. 1. Induction of autophagy in the HCV replicon cells. (A) The starved Huh7 cells and HCV replicon cells harboring a sub- or full genomic RNA of strain Con1 or strain JFH1 were subjected to immunoblotting using the appropriate antibodies. The asterisk indicates a nonspecific band. (B) Subcellular localizations of LC3 and NS5A were determined by confocal microscopy. The replicon cells and the starved Huh7 cells were stained with DAPI and then reacted with rabbit polyclonal anti-LC3 and mouse monoclonal anti-NS5A antibodies, respectively, followed by Alexa Fluor 488- and 594-conjugated secondary antibodies, respectively. The boxed areas in the merged images are magnified. (C) SGR^{Con1} cells were treated with alpha interferon for 1 week to remove the HCV replicon RNA. The resulting cells were designated SGR^{cured} cells. The SGR^{Con1}, SGR^{cured}, and SGR^{JEV} cells were lysed and subjected to immunoblotting using the appropriate antibodies. (D) Subcellular localization of LC3 and JEV NS3 and HCV NS5A was determined by confocal microscopy after staining with DAPI, followed by staining with rabbit polyclonal anti-LC3 and anti-JEV NS3 antibodies and mouse monoclonal anti-NS5A antibodies and then with the appropriate secondary antibodies. The data shown are representative of three independent experiments.

cells by treatment with the inhibitors, whereas only a slight increase was observed in the SGR^{Con1} cells (5.4-fold versus 1.6-fold) (Fig. 2A), suggesting that autophagy is suppressed in the HCV replicon cells. Furthermore, cytoplasmic accumulation of LC3 was significantly increased in the naïve Huh7 cells by treatment with the inhibitors, in contrast to the only slight increase induced by treatment in the SGR^{Con1} cells (Fig. 2B). In SGR^{Con1} cells, the LC3 foci were colocalized with the poly-

ubiquitin-binding protein p62/SQSTM1, a specific substrate for autophagy (18), suggesting that most of the autophagosomes were distributed in the cytoplasm of the SGR^{Con1} cells (Fig. 2B and C). Next, to examine the autophagy flux in the SGR^{Con1} cells, we monitored the green fluorescent protein (GFP)-conjugated LC3 dynamics in living cells by using time-lapse imaging techniques (see movies in the supplemental material). A large number of small GFP-LC3 foci were detected in the

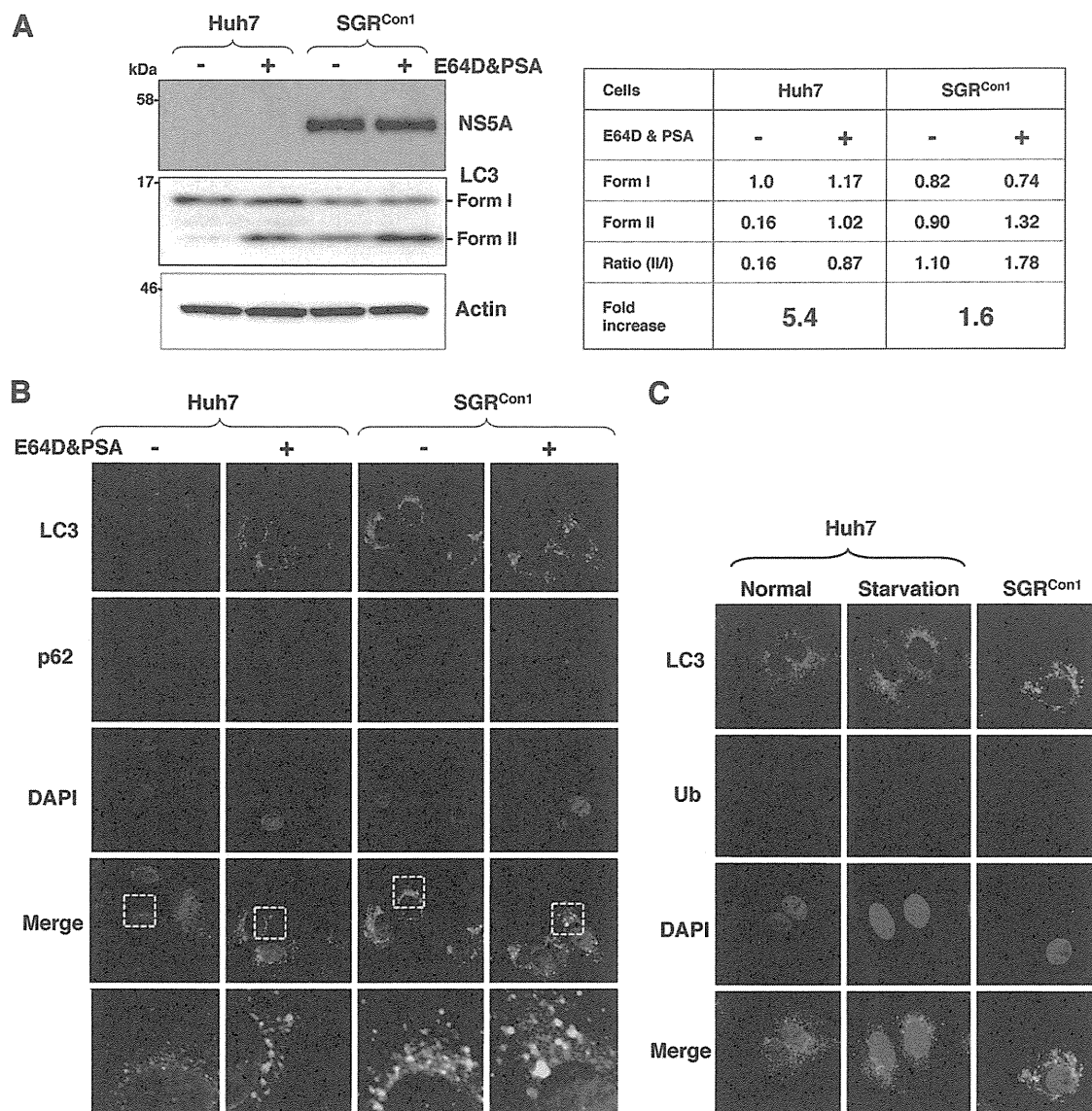


FIG. 2. Autophagy flux is impaired in the HCV replicon cells. Autophagy flux assay using lysosomal protease inhibitors. (A) Huh7 and SGR^{Con1} cells were treated with 20 μ M E64D and pepstatin A (PSA) for 6 h, and the cell lysates were subjected to immunoblotting. The density of the protein band was estimated by Multi Gauge version 2.2 (Fujifilm). (B) After nuclear staining with DAPI, the intracellular localizations of LC3 and p62 in each cell were determined by staining with rabbit polyclonal anti-LC3 and mouse monoclonal anti-62 antibodies, respectively, followed by staining with Alexa Fluor 488- and 594-conjugated secondary antibodies, respectively. The resulting cells were observed by confocal microscopy. (C) Colocalization of accumulated LC3 with ubiquitinated proteins (Ub) in SGR^{Con1} cells. Nontreated and starved Huh7 cells and SGR^{Con1} cells were fixed and stained with DAPI and rabbit anti-LC3 and anti-ubiquitin (6C1.17) (BD) polyclonal antibodies, respectively, and then with the appropriate secondary antibodies. Subcellular localizations of LC3 and Ub were determined by confocal microscopy. The data shown are representative of three independent experiments.

starved Huh7 cell, moved quickly, and finally disappeared within 30 min. Although small foci of GFP-LC3 exhibited characteristics similar to those in the starved cells, some large foci exhibited confined movement and maintained constant fluorescence for at least 3 h in the SGR^{Con1} cells. The GFP-LC3 foci in the SGR^{JFH1} cells showed characteristics similar to those in the starved cells. These results support the notion that autophagy flux is suppressed in the SGR^{Con1} cells at some step after autophagosome formation.

Impairment of autolysosomal acidification causes incomplete autophagy in the replicon cell of strain Con1. Recent

studies have shown that some viruses inhibit the autophagy pathway by blocking the autolysosome formation (10, 42). Therefore, we determined the autolysosome formation in the HCV replicon cells through the fusion of autophagosome with lysosome. Colocalization of small foci of LC3 with LAMP1, a lysosome marker, was observed in the starved Huh7 cells, SGR^{Con1} cells, and SGR^{JFH1} cells but not in the SGR^{cured} cells (Fig. 3A), suggesting that autolysosomes are formed in the HCV replicon cells of both Con1 and JFH1 strains. The autolysosome is acidified by the vacuolar-type H⁺ ATPase (V-ATPase) and degrades substrates by the lysosomal acidic hy-

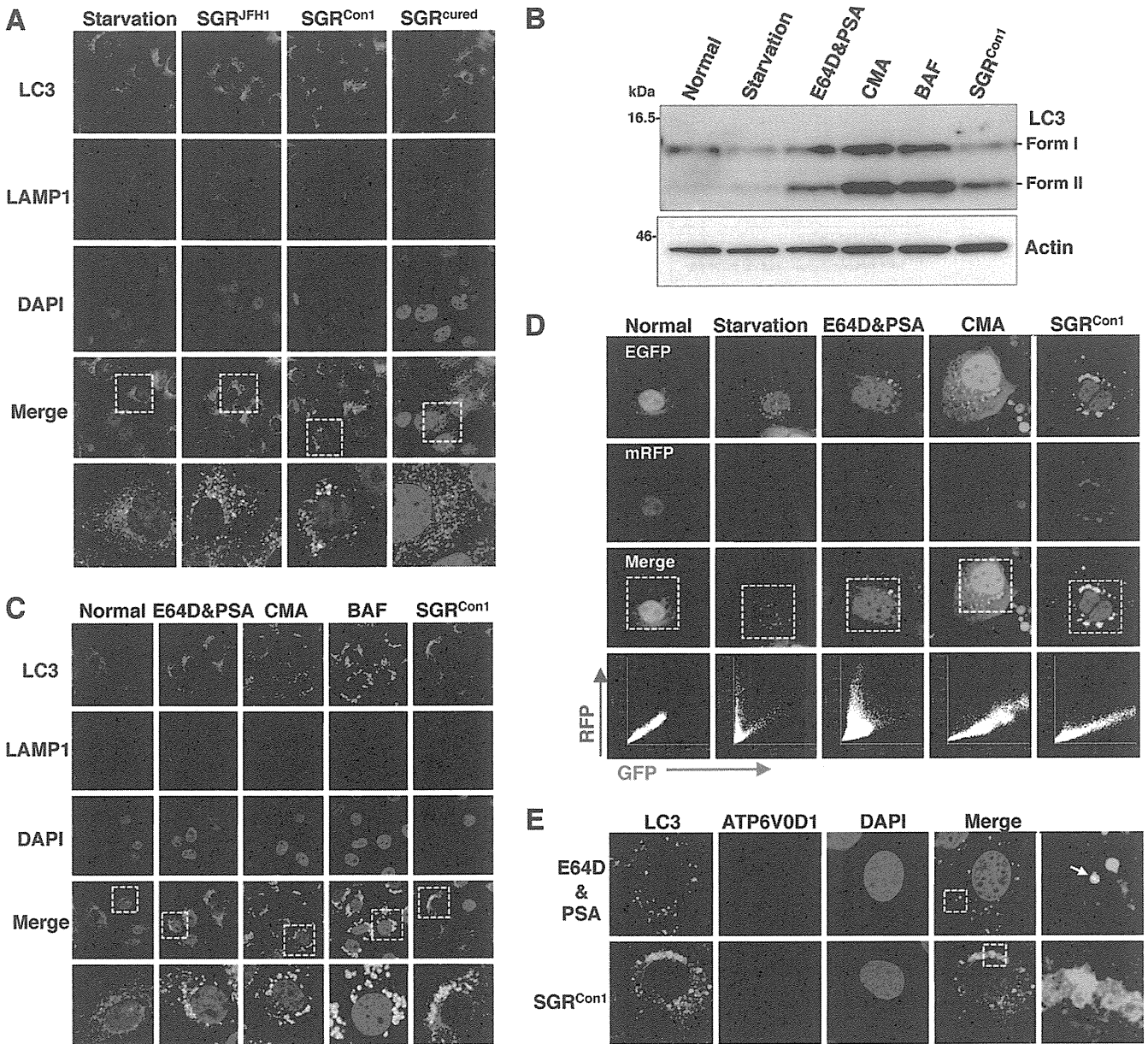


FIG. 3. Inhibition of autophagy maturation in HCV replication cells. (A) After nuclear staining with DAPI, starved Huh7 cells, replicon cells, and SGR^{cured} cells were stained with rabbit polyclonal anti-LC3 and mouse monoclonal anti-LAMP1 antibodies followed by Alexa Fluor 488- and 594-conjugated secondary antibodies, respectively, and examined by confocal microscopy. The boxed regions in the merged images are magnified. (B and C) Huh7 cells were treated with 20 μ M protease inhibitors (E64D and PSA) or a 20 nM concentration of a V-ATPase inhibitor (CMA or BAF) for 6 h. (B) Cell lysates were subjected to immunoblotting using antibodies against LC3 and β -actin. (C) Intracellular localization of LAMP1 and LC3 was determined by confocal microscopy after staining with DAPI and appropriate antibodies. The boxed areas in the merged images are magnified. (D) Tandem fluorescence-tagged LC3 assay. The expression plasmid encoding mRFP-GFP-tandem-tagged LC3 was transfected into naïve and starved Huh7 cells or into the SGR^{Con1} cells treated with the indicated inhibitors at 36 h posttransfection. The resulting cells were fixed at 42 h posttransfection, and the relative GFP and RFP signals were determined by confocal microscopy. The fluorescent values in the boxes of the merged images were determined and shown as dot plots in the bottom column of the grid, in which the *x* and *y* axes indicate the signals of GFP and RFP, respectively. (E) Huh7 cells treated with E64D and PSA and the SGR^{Con1} cells were stained with DAPI and then with rabbit polyclonal anti-LC3 and mouse monoclonal anti-ATP6V0D1 antibodies followed by Alexa Fluor 488- and 594-conjugated secondary antibodies, respectively. The boxed regions in the merged images are magnified. A white arrow indicates colocalization of LC3 and ATP6V0D1. The data shown are representative of three independent experiments.

drolases in the vesicle (2). Next, to determine the possibility of a deficiency in the acidification of the autolysosome on the autophagic dysfunction in the Con1 replicon cells, Huh7 cells were treated with the protease inhibitors E64D and pepstatin

A (PSA) or with each of the V-ATPase inhibitors concanamycin A (CMA) and bafilomycin A1 (BAF). The amount of LC3-II was significantly increased in Huh7 cells treated with the inhibitors just as in the SGR^{Con1} cells (Fig. 3B). Further-

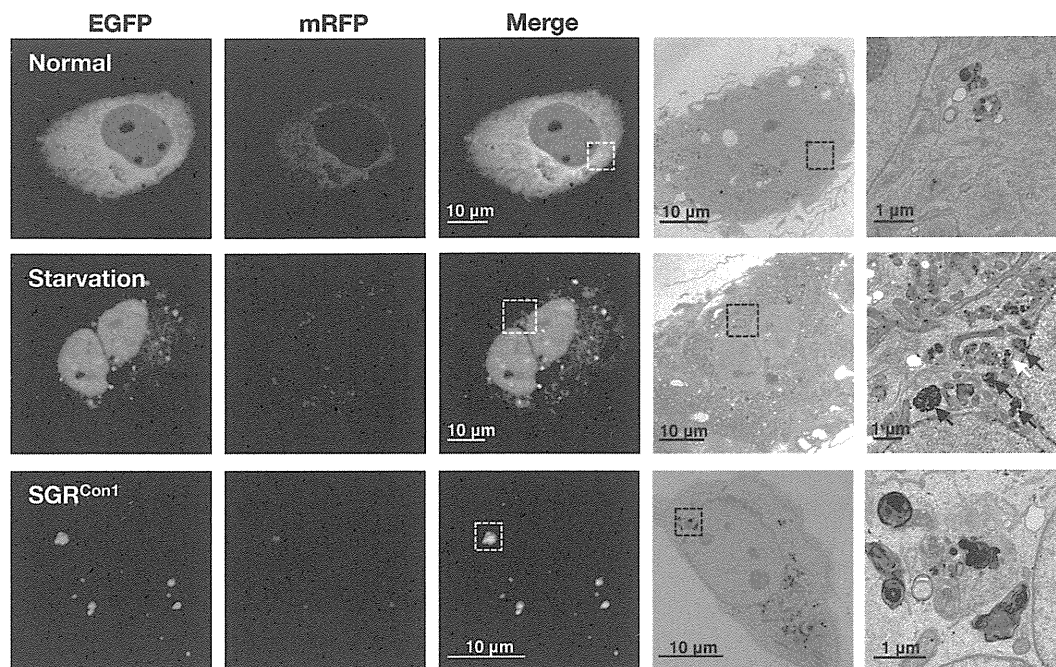


FIG. 4. Correlative fluorescence microscopy-electron microscopy (FM-EM) analysis. The expression plasmid encoding mRFP-GFP-tandem-tagged LC3 was transfected into naïve and starved Huh7 cells or into the SGR^{Con1} cells as described in the legend to Fig. 3D, and the mRFP-GFP-tandem-tagged LC3 signals were observed at 36 h posttransfection. The boxed regions in the merged images are magnified. The data shown are representative of three independent experiments.

more, the large foci of LC3 colocalized with LAMP1 appeared in the cells treated with the V-ATPase inhibitors, as seen in SGR^{Con1} cells (Fig. 3C). These results suggest that stacked autophagosome flux caused by the inhibition of lysosomal degradation or acidification exhibits characteristics similar to those observed in the Con1 replicon cells.

Since the fluorescence of GFP but not that of monomeric red fluorescent protein (mRFP) disappears under the acidic environment, expression of mRFP-GFP tandem fluorescent-tagged LC3 (tfLC3) is capable of being used to monitor the acidic status of the autolysosome (24). Both GFP and mRFP fluorescent signals were unfused, some of them accumulated as small foci in Huh7 cells after starvation or by treatment with the protease inhibitors, and half of the foci of mRFP were not colocalized with those of GFP (Fig. 3D), indicating that half of the foci are in an acidic state due to maturation into an autolysosome after fusion with a lysosome. On the other hand, the large foci of GFP and mRFP were completely colocalized in Huh7 cells treated with CMA or in the SGR^{Con1} cells. These results suggest that the large foci of LC3 in the SGR^{Con1} cells are not under acidic conditions. Recently, it was shown that the lack of lysosomal acidification in human genetic disorders due to dysfunction in assembly/sorting of V-ATPase induces incomplete autophagy similar to that observed in SGR^{Con1} cells (31, 45). Therefore, to explore the reason for the lack of acidification of the autolysosome in the SGR^{Con1} cells, we examined the subcellular localization of ATP6V0D1, a subunit of the integral membrane V₀ complex of V-ATPase. Colocalization of ATP6V0D1 with large foci of LC3 was observed in Huh7 cells treated with the protease inhibitors but not in SGR^{Con1} cells (Fig. 3E), suggesting that dislocation of V-

ATPase may participate in the impairment of the autolysosomal acidification in the SGR^{Con1} cells.

We further examined the morphological characteristics of the LC3-positive compartments by using correlative fluorescence microscopy-electron microscopy (FM-EM) (Fig. 4). The starved Huh7 cells exhibited a small double-membrane vesicle (white arrow) and high-density single-membrane structures (black arrows) in close proximity to the correlative position of the GFP- and mRFP-positive LC3 compartments, which are considered to be the autophagosome and lysosome/autolysosome, respectively. In contrast, many high-density membranous structures were detected in the correlative position of the large GFP- and mRFP-positive LC3 compartment in the SGR^{Con1} cells, which is well consistent with the observation in the time-lapse imaging in which small foci of LC3 headed toward and assembled with the large LC3-positive compartment (see movies in the supplemental material). These results suggest that the formation of large aggregates with aberrant inner structures in the SGR^{Con1} cells may impair maturation of the autolysosome through the interference of further fusion with functional lysosomes for the degradation.

The secretion of immature cathepsin B is enhanced in the replicon cell of strain Con1. Lysosomal acidification is required for the cleavage of cathepsins for activation, and cathepsin B (CTSB) is processed under acidic conditions (13). Although a marginal decrease of CTSB was detected in the whole lysates of the SGR^{Con1} cells, a significant reduction in the expression of both unprocessed (pro-CTSB) and matured CTSB was observed in the lysosomal fractions of the SGR^{Con1} cells compared with those of the naïve Huh7 and the SGR^{cured} cells (Fig. 5A). LAMP1 was concentrated at a similar level in

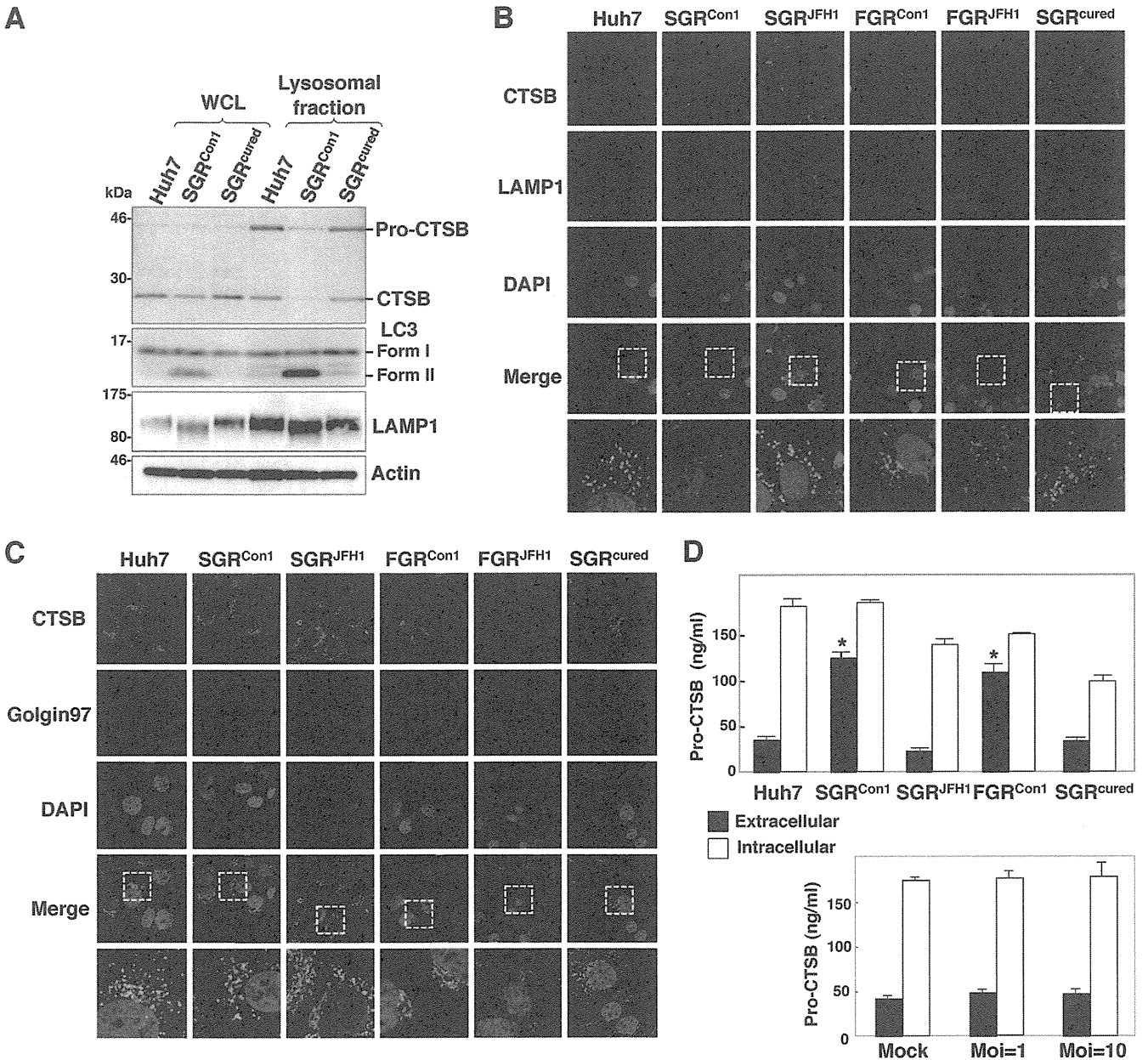


FIG. 5. Enhanced secretion of pro-CTSB in the HCV replicon cells. (A) The whole-cell lysate (WCL) and lysosomal fraction prepared from Huh7, SGR^{Con1}, and SGR^{cured} cells were subjected to immunoblotting. (B and C) Huh7 cells, HCV replicon cells, and SGR^{cured} cells were stained with DAPI, rabbit polyclonal anti-CTSB antibody, and mouse anti-LAMP1 (B) or anti-Golgin97 (C) antibody. The boxed areas in the merged images are magnified. (D) Expression of pro-cathepsin B in the culture supernatants (black bars) and cell lysates (white bars) of the Huh7, SGR^{Con1}, SGR^{JFH1}, FGR^{Con1}, and SGR^{cured} cells and the SGR^{cured} cells infected with HCVec at a multiplicity of infection (Moi) of 1 or 10 and incubated for 72 h was determined by enzyme-linked immunosorbent assay (ELISA). The error bars indicate standard deviations. The asterisks indicate significant differences ($P < 0.01$) versus the control value. The data shown are representative of three independent experiments.

the lysosomal fractions of the cells, whereas LC-II was detected in the fractions of the SGR^{Con1} cells but not in those of Huh7 and the SGR^{cured} cells, suggesting that autophagosomes and/or autolysosomes in the SGR^{Con1} cells are fractionated in the lysosomal fraction. Colocalization of CTSB with LAMP1 was observed in the naïve Huh7 cells, in the SGR^{cured} cells, and in the replicon cells harboring a sub- or a full genomic RNA of strain JFH1 (SGR^{JFH1} and FGR^{JFH1}, respectively) but not in those of strain Con1 (SGR^{Con1} and FGR^{Con1}) (Fig. 5B). On

the other hand, CTSB was colocalized with Golgin97, a marker for the Golgi apparatus, in the SGR^{Con1} and FGR^{Con1} cells but not in other cells (Fig. 5C). Since previous reports suggested that the alkalization in the lysosome triggers secretion of the unprocessed lysosomal enzymes (19, 41), we next determined the secretion of pro-CTSB in the replicon cells. Secretion of the pro-CTSB was significantly enhanced in the replicon cells of strain Con1 but not in those of strain JFH1 and naïve and cured cells (Fig. 5D, top). Furthermore, secretion of pro-CTSB

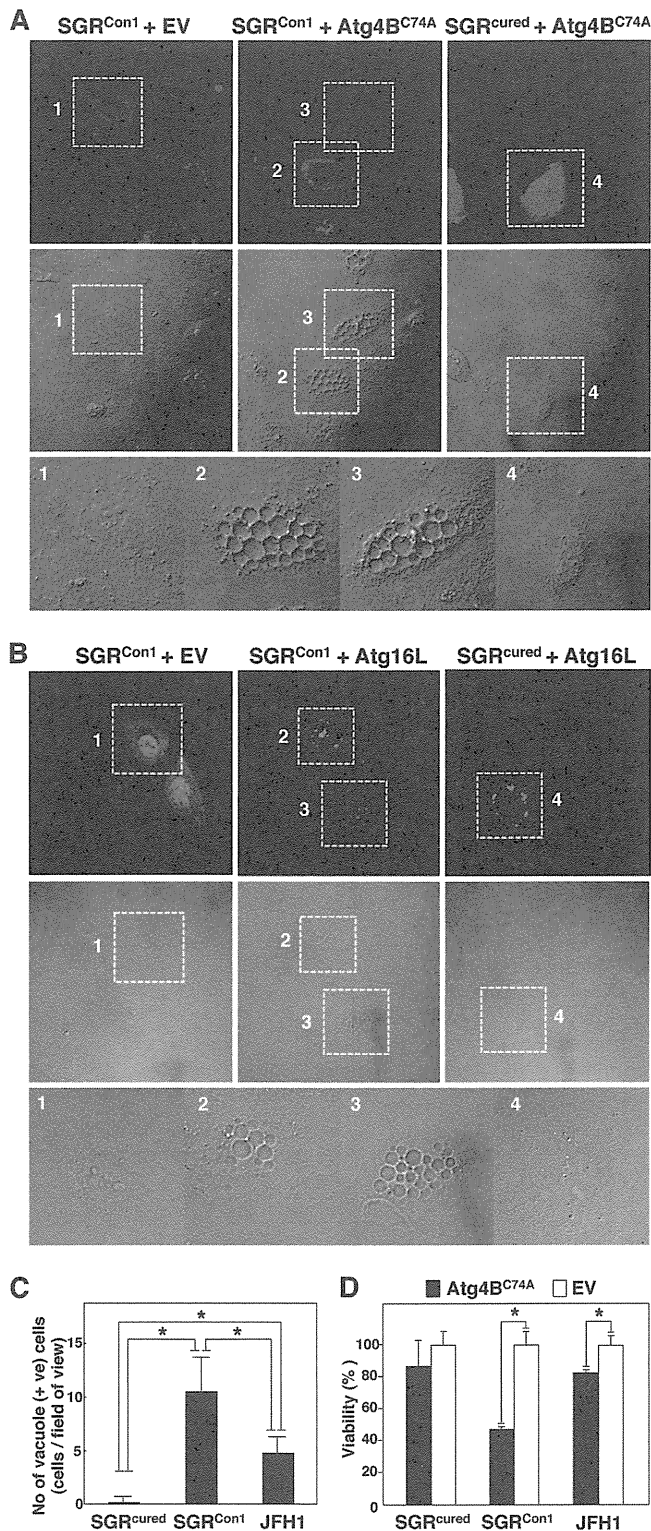


FIG. 6. Inhibition of autophagosome formation induces severe cytoplasmic vacuolations leading to cell death in the HCV replicon cells. (A) SGR^{Con1} and SGR^{cured} cells transfected with pStrawberry-Atg4B^{C74A} or empty vector pStrawberry (EV) were fixed at 48 h posttransfection and examined by fluorescence microscopy. The boxed areas in the phase-contrast images are magnified. (B) SGR^{Con1} and SGR^{cured} cells transfected with pEGFP-Atg16L or EV were examined by fluorescence microscopy at 48 h posttransfection. The boxed areas in the phase-contrast images are magnified. (C) SGR^{cured}, SGR^{Con1},

was not observed in the cured cells infected with HCVcc, an infectious HCV strain derived from strain JFH1 (Fig. 5D, bottom). Collectively, these results suggest that the dysfunction of lysosomal acidification contributes to the impairment of autophagy in the HCV replicon cells of strain Con1.

Autophagy induced in cells replicating HCV is required for cell survival. Finally, we examined the pathological significance of autophagy during HCV replication. Atg4B is known as an LC3-processing protease, and overexpression of its protease-inactive mutant (Atg4B^{C74A}) results in inhibition of the autophagosome formation (7). To our surprise, severe cytoplasmic vacuolation was observed in the SGR^{Con1} cells expressing Atg4B^{C74A} (Fig. 6A). These vacuolations were also observed in the SGR^{Con1} cells by the expression of Atg16L (Fig. 6B), a molecule that is an essential component of the autophagy complex and that, if expressed in excess amounts, can disrupt the autophagosome formation (8). Expression of Atg4B^{C74A} induced a higher level of vacuole formation in the Con1 replicon cells than in cells infected with JFH1 virus but not in the cured cells (Fig. 6C). Along with these vacuolations, cell viability was significantly decreased by the expression of Atg4B^{C74A} in SGR^{Con1} cells and slightly in JFH1 virus-infected cells (Fig. 6D). These results suggest that autophagy induced by the RNA replication of HCV is required for host cell survival.

DISCUSSION

In the present study, we demonstrated that two genotypes of HCV induce autophagy, whereas intact autophagy flux is required for the host cell to survive. The cell death characterized by cytoplasmic vacuolation that was induced in the HCV replicon cells by the inhibition of the autophagosome formation is similar to type III programmed cell death, which is distinguishable from apoptosis and autophagic cell death (4). Type III programmed cell death has been observed in the neurodegenerative diseases caused by the deposit of cytotoxic protein aggregates (15).

We previously reported that HCV hijacks chaperone complexes, which regulates quality control of proteins into the membranous web for circumventing unfolded protein response during efficient genome replication (53); in other words, the replication of HCV exacerbates the generation of proteins associated with cytotoxicity. In the experiments using a chimpanzee model, HCV of genotype 1 was successfully used to reproduce acute and chronic hepatitis similar to that in the human patients (3, 57), and transgenic mice expressing viral proteins of HCV of genotype 1b have been shown to develop

and SGR^{cured} cells infected with JFH1 virus were transfected with pStrawberry-Atg4B^{C74A}, and the number of vacuole-positive cells in each of nine fields of view was counted at 48 h posttransfection. (D) SGR^{cured}, SGR^{Con1}, and SGR^{cured} cells infected with JFH1 virus were transfected with pStrawberry-Atg4B^{C74A} (black bars) or EV (white bars), and cell viability was determined at 6 days posttransfection by using CellTiter-Glo (Promega) according to the manufacturer's protocol. The asterisks indicate significant differences ($P < 0.05$) versus the control value. The data shown are representative of three independent experiments.

Sjögren syndrome, insulin resistance, hepatic steatosis, and hepatocellular carcinoma (27, 28). In contrast, HCVcc, based on the genotype 2a strain JFH1 isolated from a patient with fulminant hepatitis C (33, 56), was unable to establish chronic infection in chimpanzees (56) or to induce cell damage and inflammation in chimeric mice xenotransplanted with human hepatocytes (17). These results imply that the onset of HCV pathogenesis could be dependent not only upon an amount but also on a property of deposited proteins, and they might explain the aggravated vacuolations under the inhibition of autophagosome formation in strain Con1 compared to that in strain JFH1. Interestingly, the overexpression of Atg4B^{C74A} or Atg16L causes eccentric cell death in the Con1 replicon cells in which autophagy flux is already disturbed. Thus, we speculated that the quarantine of undefined abnormalities endowed with high cytotoxicity by the engulfing of the autophagic membrane might be sufficient for the amelioration of HCV-induced degeneration. The autophagosomal dysfunction observed in the Con1 replicon cells may suggest that a replicant of strain Con1 was more sensitive to the lysosomal vacuolation than that of strain JFH1. Because a limitation of our study was that we were unable to use infectious HCV of other strains, it is still unclear whether the autophagic degradation can be impaired only in the replicon of HCV strain Con1 or genotype 1.

We also demonstrated that HCV replication of strain Con1 but not that of strain JFH1 facilitates the secretion of pro-CTSB. It has been well established that the secretion of pro-CTSB is enhanced in several types of tumors (26, 50). The secretion of CTSB, like the secretion of matrix metalloproteases, is a marker of the progression of the proteolytic degradation of the extracellular matrix, which plays an important part in cancer invasion and metastasis. Since infection with HCV of genotype 1 is clinically considered a risk factor for the development of hepatocellular carcinoma (14, 51), the enhanced secretion of pro-CTSB by the replication of genotype 1 strains might synergistically promote infiltration of hepatocellular carcinoma.

As shown elsewhere (see movies in the supplemental material), although most degradations of the autophagosome were impaired due to a dislocalization of a V-ATPase subunit, some autophagic degradation was achieved in the SGR^{Con1} cells similar to that in the starved Huh7 cells. Moreover, the stagnated autophagy flux was rescued by the treatment of alpha interferon accompanied by elimination of HCV (Fig. 1C and D). Interestingly, we observed neither a significant impairment of lysosomal degradation nor the intracellular activity of cathepsins in the replicon cells of HCV strain Con1 (data not shown). Therefore, there might be a specific dysfunction within the autolysosome during the replication of HCV strain Con1. Detailed studies are needed to elucidate how HCV strain Con1 disturbs the sorting of V-ATPase.

A close relationship between autophagy and the immune system has been gradually unveiled (47). Autophagy assists not only in the direct elimination of pathogens by hydrolytic degradation but also in antigen processing in antigen-presenting cells such as macrophage and dendritic cells (DC) for presentation by major histocompatibility complex (MHC) I and II (11). Moreover, autophagy plays important roles in T lymphocyte homeostasis (44). As such, in some instances, interruptions of autophagy can allow microorganisms to escape from

the host immune system. Indeed, the immune response against herpes simplex virus was suppressed by blocking the autophagy (6). With regard to HCV, functionally impaired DC dysfunctions marked by poor DC maturation, impaired antigen presentation, and attenuated cytokine production have been reported in tissue culture models and chronic hepatitis C patients (1, 22, 46). In addition, reduction of cell surface expression of MHC-I in HCV genotype 1b replicon cells has been reported (55). We confirmed that levels of cell surface expression of MHC-I in the replicon cells of genotype 1b, but not of genotype 2a, were reduced in comparison with those in the cured cells (data not shown). Hence it might be feasible to speculate that the replication of HCV RNA of genotype 1 induces an incomplete autophagy for attenuating antigen presentation to establish persistent infection. In contrast, autophagy is known to serve as a negative regulator of innate immunity (21, 54). A recent report demonstrated that autophagy induced by infection with strain JFH1 or dengue virus attenuates innate immunity to promote viral replication (23), indicating that an HCV genotype 2a strain may facilitate autophagy to evade innate immunity.

In this study, we demonstrated that HCV utilizes autophagy to circumvent the cell death induced by vacuole formation for its survival. This unique strategy of HCV propagation may provide new clues to the virus-host interaction and, ultimately, to the pathogenesis of infection by various genotypes of HCV.

ACKNOWLEDGMENTS

We thank H. Murase and M. Tomiyama for their secretarial work. We also thank R. Bartenschlager and T. Wakita for providing cell lines and plasmids.

This work was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare (Research on Hepatitis), the Ministry of Education, Culture, Sports, Science, and Technology, and the Osaka University Global Center of Excellence Program.

REFERENCES

1. **Auffermann-Gretzinger, S., E. B. Keefe, and S. Levy.** 2001. Impaired dendritic cell maturation in patients with chronic, but not resolved, hepatitis C virus infection. *Blood* **97**:3171–3176.
2. **Beyenbach, K. W., and H. Wiczorek.** 2006. The V-type H⁺ ATPase: molecular structure and function, physiological roles and regulation. *J. Exp. Biol.* **209**:577–589.
3. **Bradley, D. W.** 2000. Studies of non-A, non-B hepatitis and characterization of the hepatitis C virus in chimpanzees. *Curr. Top. Microbiol. Immunol.* **242**:1–23.
4. **Clarke, P. G.** 1990. Developmental cell death: morphological diversity and multiple mechanisms. *Anat. Embryol. (Berl.)* **181**:195–213.
5. **Dreux, M., P. Gastaminza, S. F. Wieland, and F. V. Chisari.** 2009. The autophagy machinery is required to initiate hepatitis C virus replication. *Proc. Natl. Acad. Sci. U. S. A.* **106**:14046–14051.
6. **English, L., et al.** 2009. Autophagy enhances the presentation of endogenous viral antigens on MHC class I molecules during HSV-1 infection. *Nat. Immunol.* **10**:480–487.
7. **Fujita, N., et al.** 2008. An Atg4B mutant hampers the lipidation of LC3 paralogs and causes defects in autophagosome closure. *Mol. Biol. Cell* **19**:4651–4659.
8. **Fujita, N., et al.** 2008. The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy. *Mol. Biol. Cell* **19**:2092–2100.
9. **Fujitani, Y., C. Ebato, T. Uchida, R. Kawamori, and H. Watada.** 2009. β -cell autophagy: a novel mechanism regulating β -cell function and mass: lessons from β -cell-specific Atg7-deficient mice. *Islets* **1**:151–153.
10. **Gannage, M., et al.** 2009. Matrix protein 2 of influenza A virus blocks autophagosome fusion with lysosomes. *Cell Host Microbe* **6**:367–380.
11. **Gannage, M., and C. Munz.** 2009. Autophagy in MHC class II presentation of endogenous antigens. *Curr. Top. Microbiol. Immunol.* **335**:123–140.
12. **Hara, T., et al.** 2006. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature* **441**:885–889.
13. **Hasilik, A.** 1992. The early and late processing of lysosomal enzymes: proteolysis and compartmentation. *Experientia* **48**:130–151.

14. Hatzakis, A., et al. 1996. Hepatitis C virus 1b is the dominant genotype in HCV-related carcinogenesis: a case-control study. *Int. J. Cancer* **68**:51–53.
15. Hirabayashi, M., et al. 2001. VCP/p97 in abnormal protein aggregates, cytoplasmic vacuoles, and cell death, phenotypes relevant to neurodegeneration. *Cell Death Differ.* **8**:977–984.
16. Hiraga, N., et al. 2011. Rapid emergence of telaprevir resistant hepatitis C virus strain from wildtype clone in vivo. *Hepatology* (Baltimore, Md.) **54**: 781–788.
17. Hiraga, N., et al. 2007. Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis C virus and its susceptibility to interferon. *FEBS Lett.* **581**:1983–1987.
18. Ichimura, Y., E. Kominami, K. Tanaka, and M. Komatsu. 2008. Selective turnover of p62/A170/SQSTM1 by autophagy. *Autophagy* **4**:1063–1066.
19. Isidoro, C., et al. 1995. Altered intracellular processing and enhanced secretion of procathepsin D in a highly deviated rat hepatoma. *Int. J. Cancer* **60**:61–64.
20. Jacobson, I. M., P. Cacoub, L. Dal Maso, S. A. Harrison, and Z. M. Younossi. 2010. Manifestations of chronic hepatitis C virus infection beyond the liver. *Clin. Gastroenterol. Hepatol.* **8**:1017–1029.
21. Jounai, N., et al. 2007. The Atg5 Atg12 conjugate associates with innate antiviral immune responses. *Proc. Natl. Acad. Sci. U. S. A.* **104**:14050–14055.
22. Kanto, T., et al. 1999. Impaired allostimulatory capacity of peripheral blood dendritic cells recovered from hepatitis C virus-infected individuals. *J. Immunol.* **162**:5584–5591.
23. Ke, P. Y., and S. S. Chen. 2011. Activation of the unfolded protein response and autophagy after hepatitis C virus infection suppresses innate antiviral immunity in vitro. *J. Clin. Invest.* **121**:37–56.
24. Kimura, S., N. Fujita, T. Noda, and T. Yoshimori. 2009. Monitoring autophagy in mammalian cultured cells through the dynamics of LC3. *Methods Enzymol.* **452**:1–12.
25. Kiyosawa, K., et al. 1990. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. *Hepatology* **12**:671–675.
26. Koblinski, J. E., et al. 2002. Interaction of human breast fibroblasts with collagen I increases secretion of procathepsin B. *J. Biol. Chem.* **277**:32220–32227.
27. Koike, K., et al. 1997. Sialadenitis histologically resembling Sjogren syndrome in mice transgenic for hepatitis C virus envelope genes. *Proc. Natl. Acad. Sci. U. S. A.* **94**:233–236.
28. Koike, K., T. Tsutsumi, H. Yotsuyanagi, and K. Moriya. 2010. Lipid metabolism and liver disease in hepatitis C viral infection. *Oncology* **78**(Suppl. 1):24–30.
29. Komatsu, M., et al. 2006. Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature* **441**:880–884.
30. Komatsu, M., et al. 2007. Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. *Cell* **131**:1149–1163.
31. Lee, J. H., et al. 2010. Lysosomal proteolysis and autophagy require presenilin 1 and are disrupted by Alzheimer-related PS1 mutations. *Cell* **141**: 1146–1158.
32. Levine, B., and G. Kroemer. 2008. Autophagy in the pathogenesis of disease. *Cell* **132**:27–42.
33. Lindenbach, B. D., et al. 2005. Complete replication of hepatitis C virus in cell culture. *Science* **309**:623–626.
34. Lohmann, V., et al. 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285**:110–113.
35. Manns, M. P., et al. 2001. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* **358**:958–965.
36. McHutchison, J. G., et al. 2009. Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. *N. Engl. J. Med.* **360**:1827–1838.
37. Mizushima, N. 2007. Autophagy: process and function. *Genes Dev.* **21**:2861–2873.
38. Moradpour, D., F. Penin, and C. M. Rice. 2007. Replication of hepatitis C virus. *Nat. Rev. Microbiol.* **5**:453–463.
39. Moriishi, K., and Y. Matsuura. 2007. Host factors involved in the replication of hepatitis C virus. *Rev. Med. Virol.* **17**:343–354.
40. Moriishi, K., and Y. Matsuura. 2003. Mechanisms of hepatitis C virus infection. *Antivir. Chem. Chemother.* **14**:285–297.
41. Oda, K., Y. Nishimura, Y. Ikehara, and K. Kato. 1991. Bafilomycin A1 inhibits the targeting of lysosomal acid hydrolases in cultured hepatocytes. *Biochem. Biophys. Res. Commun.* **178**:369–377.
42. Orvedahl, A., et al. 2007. HSV-1 ICP34.5 confers neurovirulence by targeting the Beclin 1 autophagy protein. *Cell Host Microbe* **1**:23–35.
43. Poordad, F., et al. 2011. Boceprevir for untreated chronic HCV genotype 1 infection. *N. Engl. J. Med.* **364**:1195–1206.
44. Pua, H. H., I. Dzhagalov, M. Chuck, N. Mizushima, and Y. W. He. 2007. A critical role for the autophagy gene Atg5 in T cell survival and proliferation. *J. Exp. Med.* **204**:25–31.
45. Ramachandran, N., et al. 2009. VMA21 deficiency causes an autophagic myopathy by compromising V-ATPase activity and lysosomal acidification. *Cell* **137**:235–246.
46. Saito, K., et al. 2008. Hepatitis C virus inhibits cell surface expression of HLA-DR, prevents dendritic cell maturation, and induces interleukin-10 production. *J. Virol.* **82**:3320–3328.
47. Schmid, D., and C. Munz. 2007. Innate and adaptive immunity through autophagy. *Immunity* **27**:11–21.
48. Schutte, K., J. Bornschein, and P. Malfertheiner. 2009. Hepatocellular carcinoma—epidemiological trends and risk factors. *Dig. Dis.* **27**:80–92.
49. Sir, D., et al. 2008. Induction of incomplete autophagic response by hepatitis C virus via the unfolded protein response. *Hepatology* **48**:1054–1061.
50. Sloane, B. F., et al. 2005. Cathepsin B and tumor proteolysis: contribution of the tumor microenvironment. *Semin. Cancer Biol.* **15**:149–157.
51. Stankovic-Djordjevic, D., et al. 2007. Hepatitis C virus genotypes and the development of hepatocellular carcinoma. *J. Dig. Dis.* **8**:42–47.
52. Strader, D. B., T. Wright, D. L. Thomas, and L. B. Seeff. 2004. Diagnosis, management, and treatment of hepatitis C. *Hepatology* **39**:1147–1171.
53. Taguwa, S., et al. 2009. Cochaperone activity of human butyrate-induced transcript 1 facilitates hepatitis C virus replication through an Hsp90-dependent pathway. *J. Virol.* **83**:10427–10436.
54. Tal, M. C., et al. 2009. Absence of autophagy results in reactive oxygen species-dependent amplification of RLR signaling. *Proc. Natl. Acad. Sci. U. S. A.* **106**:2770–2775.
55. Tardif, K. D., and A. Siddiqui. 2003. Cell surface expression of major histocompatibility complex class I molecules is reduced in hepatitis C virus subgenomic replicon-expressing cells. *J. Virol.* **77**:11644–11650.
56. Wakita, T., et al. 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat. Med.* **11**:791–796.
57. Walker, C. M. 1997. Comparative features of hepatitis C virus infection in humans and chimpanzees. *Springer Semin. Immunopathol.* **19**:85–98.
58. Wasley, A., and M. J. Alter. 2000. Epidemiology of hepatitis C: geographic differences and temporal trends. *Semin. Liver Dis.* **20**:1–16.
59. Wong, J., et al. 2008. Autophagosome supports coxsackievirus B3 replication in host cells. *J. Virol.* **82**:9143–9153.
60. Yoshimori, T., and T. Noda. 2008. Toward unraveling membrane biogenesis in mammalian autophagy. *Curr. Opin. Cell Biol.* **20**:401–407.

Elimination of Hepatitis C Virus from Hepatocytes by a Selective Activation of Therapeutic Molecules

Xiaoyu Wen¹*, Takayuki Abe¹*, Hiroshi Kukihara¹, Shuhei Taguwa¹, Yoshio Mori¹, Hideki Tani¹, Nobuyuki Kato², Tetsuro Suzuki³, Masashi Tatsumi⁴, Kohji Moriishi¹, Yoshiharu Matsuura¹*

1 Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan, **2** Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan, **3** Department of Infectious Diseases, Hamamatsu University School of Medicine, Hamamatsu, Japan, **4** AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan

Abstract

To eliminate hepatitis C virus (HCV) from infected hepatocytes, we generated two therapeutic molecules specifically activated in cells infected with HCV. A dominant active mutant of interferon (IFN) regulatory factor 7 (IRF7) and a negative regulator of HCV replication, VAP-C (Vesicle-associated membrane protein-associated protein subtype C), were fused with the C-terminal region of IPS-1 (IFN β promoter stimulator-1), which includes an HCV protease cleavage site that was modified to be localized on the ER membrane, and designated cIRF7 and cVAP-C, respectively. In cells expressing the HCV protease, cIRF7 was cleaved and the processed fragment was migrated into the nucleus, where it activated various IFN promoters, including promoters of IFN α 6, IFN β , and IFN stimulated response element. Activation of the IFN promoters and suppression of viral RNA replication were observed in the HCV replicon cells and in cells infected with the JFH1 strain of HCV (HCVcc) by expression of cIRF7. Suppression of viral RNA replication was observed even in the IFN-resistant replicon cells by the expression of cIRF7. Expression of the cVAP-C also resulted in suppression of HCV replication in both the replicon and HCVcc infected cells. These results suggest that delivery of the therapeutic molecules into the liver of hepatitis C patients, followed by selective activation of the molecules in HCV-infected hepatocytes, is a feasible method for eliminating HCV.

Citation: Wen X, Abe T, Kukihara H, Taguwa S, Mori Y, et al. (2011) Elimination of Hepatitis C Virus from Hepatocytes by a Selective Activation of Therapeutic Molecules. PLoS ONE 6(1): e15967. doi:10.1371/journal.pone.0015967

Editor: Paul Digard, University of Cambridge, United Kingdom

Received: September 29, 2010; **Accepted:** December 7, 2010; **Published:** January 6, 2011

Copyright: © 2011 Wen et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare; the Ministry of Education, Culture, Sports, Science, and Technology; the Osaka University Global Center of Excellence Program; and the Foundation for Biomedical Research and Innovation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: matsuura@biken.osaka-u.ac.jp

† These authors contributed equally to this work.

Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver diseases. A high risk of chronicity is the major concern of HCV infection, since chronic HCV infection often leads to liver cirrhosis and hepatocellular carcinoma [1,2]. Although the proportion of patients achieving a sustained virological response (SVR) has been increased by the recent use of combination therapy with pegylated-interferon- α (PEG-IFN α) and ribavirin (RBV), half of patients still exhibit no response to this therapy, suggesting that the IFN signaling pathway is modulated by HCV infection. In addition, various side effects have been reported in more than 20% of patients treated with this combination therapy [3].

HCV belongs to the family *Flaviviridae* and possesses a single positive-stranded RNA genome that encodes a single polyprotein composed of about 3,000 amino acids. The HCV polyprotein is processed into 10 viral proteins by host and viral proteases. Viral structural proteins, including the capsid protein and two envelope proteins, are located in the N-terminal one third of the polyprotein, followed by nonstructural proteins. The NS2 protease cleaves its own carboxyl terminus and NS3 cleaves the downstream positions to produce NS4A, NS4B, NS5A and NS5B. Although laboratory strains of HCV propagating in cell culture (HCVcc) have been established based on the full-length genome of the

genotype 2a JFH1 strain [4], establishment of a robust cell culture system capable of propagating serum-derived HCV from hepatitis C patients has not yet been achieved.

Type I IFN exhibits potent antiviral effects through the regulation of hundreds of IFN-stimulated genes (ISGs) which encode proteins involved in the establishment of antiviral state in cells [5]. IFNs induce transcription of ISGs through activation of the Jak-STAT pathway [6]. Binding of type I IFN to the IFN receptor induces phosphorylation of the receptor-associated tyrosine kinases, Jak1 and Tyk2, and then these kinases activate STAT1 and STAT2. The phosphorylated STATs migrate into the nucleus and activate ISG promoters through binding to the specific responsible elements. HCV infection has been suggested to impair the IFN production through multiple pathways. The IFN-induced Jak-STAT signaling is inhibited in cells and transgenic mice expressing HCV proteins and in the liver biopsy samples of chronic hepatitis C patients [7–9].

Induction of type I IFN upon infection with pathogens is crucial for innate immunity, and it is mediated by the activation of pattern-recognition receptors, including Toll-like receptors (TLRs) and cytosolic receptors, such as RIG-I and MDA5 [10–12]. The induction of type I IFN is primarily controlled at the gene transcriptional level, wherein a family of transcription factors known as IFN regulatory factors (IRFs) play a pivotal role. IRF3