The American Journal of Pathology, Vol. 175, No. 4, October 2009

DOI: 10.2353/ajpath.2009.090102

Immunopathology and Infectious Diseases

Tacrolimus Ameliorates Metabolic Disturbance and Oxidative Stress Caused by Hepatitis C Virus Core Protein

Analysis Using Mouse Model and Cultured Cells

Kyoji Moriya,* Hideyuki Miyoshi,* Takeya Tsutsumi,* Seiko Shinzawa,* Hajime Fujie,* Yoshizumi Shintani,* Hiroshi Yotsuyanagi,* Kohji Moriishi,† Yoshiharu Matsuura,† Tetsuro Suzuki,‡ Tatsuo Miyamura,‡ and Kazuhiko Koike*

From the Department of Internal Medicine, Graduate School of Medicine, University of Tokyo, Tokyo; the Department of Molecular Virology,† Research Institute for Microbial Diseases, Osaka University, Osaka; and the Department of Virology II,‡ National Institute of Infectious Diseases, Tokyo, Japan*

Hepatic steatosis and insulin resistance are factors that aggravate the progression of liver disease caused by hepatitis C virus (HCV) infection. In the pathogenesis of liver disease and metabolic disorders in HCV infection, oxidative stress due to mitochondrial respiratory chain dysfunction plays a pivotal role. Tacrolimus (FK506) is supposed to protect mitochondrial respiratory function. We studied whether tacrolimus affects the development of HCV-associated liver disease using *HCV core gene* transgenic mice, which develop hepatic steatosis, insulin resistance, and hepatocellular carcinoma. Administration of tacrolimus to *HCV core gene* transgenic mice three times per week for 3 months led to a significant reduction in the amounts of lipid in the liver as well as in serum insulin. Tacrolimus treatment also ameliorated oxidative stress and DNA damage in the liver of the *core gene* transgenic mice. Tacrolimus administration reproduced these effects in a dose-dependent manner in HepG2 cells expressing the core protein. The intrahepatic level of tumor necrosis factor-α, which may be a key molecule for the pathogenesis in HCV infection, was significantly decreased in tacrolimus-treated core gene transgenic mice. Tacrolimus thus reversed the effect of the core protein in the pathogenesis of HCV-associated liver disease. 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. ***(Am J Pathol 2009, 175:1515–1524; DOI: 10.2353/ajpath.2009.090102)***

Hepatitis C virus (HCV) is a major cause of liver disease; approximately 170 million people are chronically infected worldwide. Persistent HCV infection leads to the development of chronic hepatitis, cirrhosis, and, eventually, hepatocellular carcinoma (HCC), thereby being a serious problem from both medical and socioeconomic viewpoints.[1,2] Recently, a growing amount of evidence showing that HCV infection induces alteration in lipid[3–7] and glucose metabolism has accumulated.[8,9] Augmentation of oxidative stress is also substantiated in HCV infection by a number of clinical and basic studies.[10–13]

We demonstrated previously that the core protein of HCV induces HCC in transgenic mice that have marked hepatic steatosis in the absence of inflammation.[14] In this animal model for HCV-associated HCC, there is augmentation of oxidative stress in the liver during the incubation period.[10] Also noted is an accumulation of lipid droplets that are rich with carbon 18 monounsaturated fatty acids such as oleic and vaccenic acids, which is also observed in liver tissues of patients with chronic hepatitis C com-

Supported in part by a Grant-in-Aid for Scientific Research on Priority Area from the Ministry of Education, Science, Sports and Culture of Japan, by Health Sciences research grants from the Ministry of Health, Labour and Welfare (Research on Hepatitis), and by the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Drug ADR Relief, R&D Promotion and Product Review of Japan.

Accepted for publication June 22, 2009.

Address reprint requests to Kazuhiko Koike, M.D., Ph.D., Department of Gastroenterology, Internal Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. E-mail: kkoike-tky@umin.ac.jp.

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 caster 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 μmol/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 methanolysed 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 × 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 μmol/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′-TTCCCTCCTGCAAGCTCTAC-3′ and 5′-CGCAAGAAGGTGCTAACGAAC-3′, and 5′-CCAGCAAGCTTGCAACCTTAACCA-3′ and 5′-GTAATGATCAGTCAACGGGGGAC-3′, respectively. 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 ± 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).

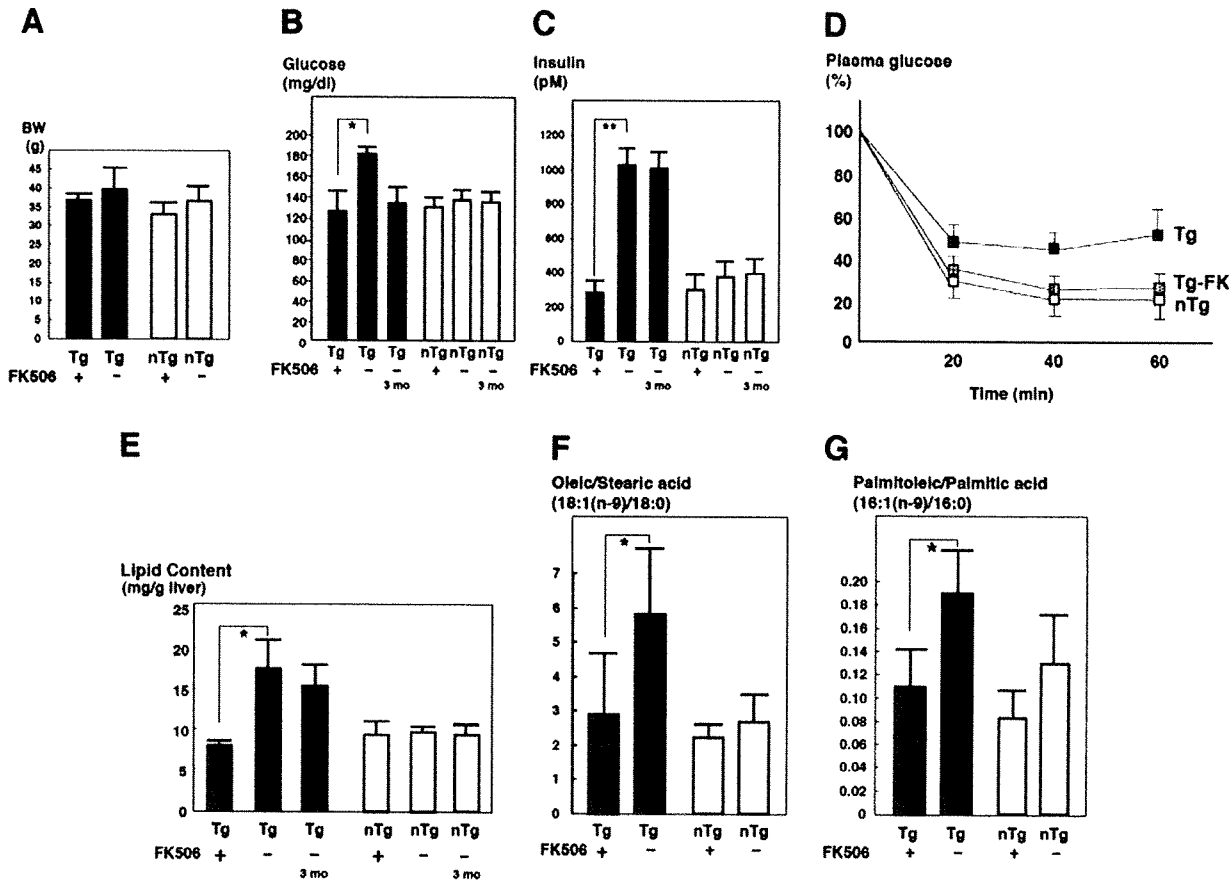

Figure 1. Effect of tacrolimus (FK506) on glucose and lipid metabolism in the core gene transgenic mice. Tacrolimus (0.1 mg/kg b.wt.) or vehicle was administered to core gene transgenic or control mice i.p., three times weekly for 3 months beginning at 3 months of age. **A:** Body weight at the baseline 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 ± 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 μmol/L for 48 hours significantly reduced the accumulation of 20:3(n-9), whereas CyA treat-

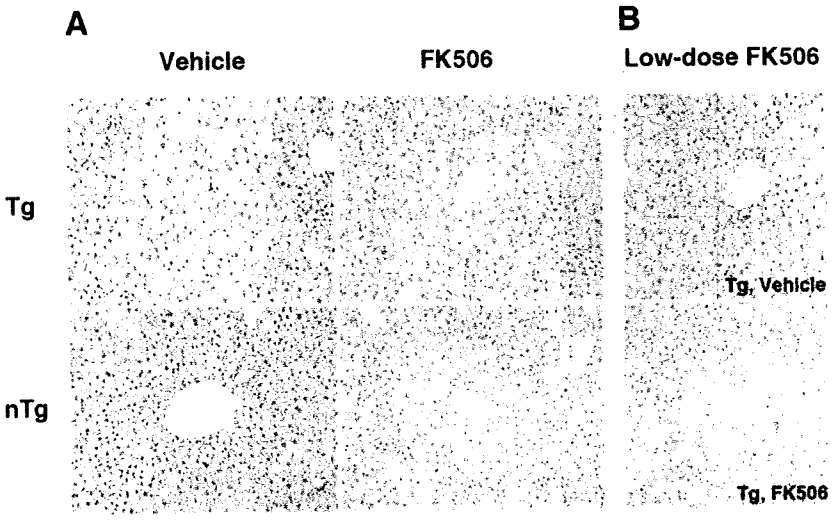

Figure 2. Morphological analysis of the liver of the core gene transgenic mice. Representative cases are shown either treated with tacrolimus (FK506) or vehicle (H&E staining). **A:** There is a prominent improvement of steatosis in the 3-month tacrolimus-treated core gene transgenic mice compared with 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, ×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/apyrimidinic site index was determined in liver tissues from the core gene transgenic mice. As shown in Figure 4B, the apurinic/apyrimidinic site index in the liver of the core gene transgenic mice, which was significantly higher

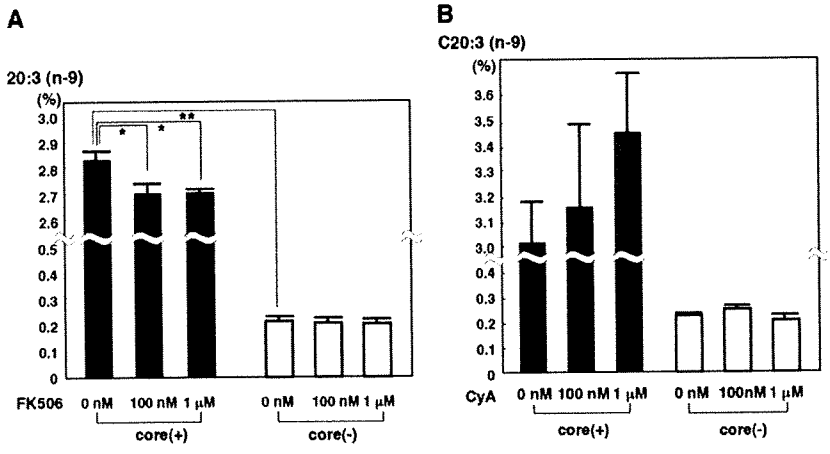

Figure 3. Effect of tacrolimus (FK506) or CyA on fatty acid compositions in HepG2 cells expressing the core protein. The fatty acid compositions of the total cell lipids were analyzed, and the percentage of 5,8,11-eicosatrienoic acid [20:3(n-9)] in the core-expressing and control HepG2 cells was calculated. **A:** Treatment with tacrolimus at 0 nmol/L, 100 nmol/L, or 1 μmol/L. **B:** Treatment with CyA at 0 nmol/L, 100 nmol/L, or 1 μmol/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 ± SE; $n = 5$ in each group. $^{*}P < 0.05$ and $^{**}P < 0.01$.

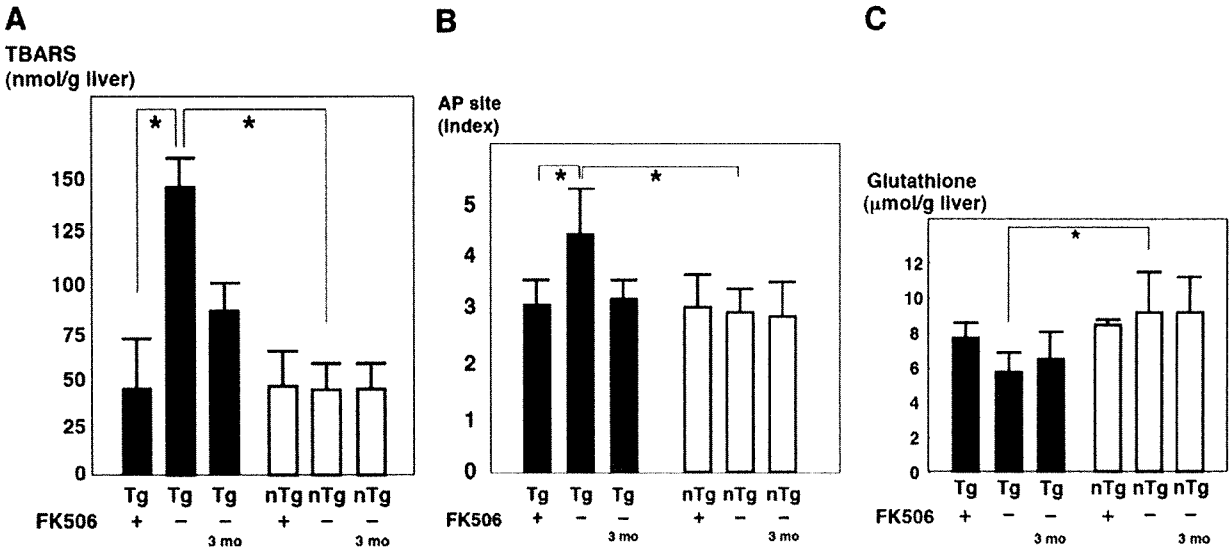

Figure 4. Effect of tacrolimus (FK506) on oxidative stress in the core gene transgenic mice. Tacrolimus (0.1 mg/kg b.wt.) or vehicle only was administered to the core gene transgenic or control mice for 3 months. **A:** Lipid peroxidation in the liver. **B:** apurinic/apyrimidinic (AP) site in the liver 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 ± 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 dysfunction. 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 μmol/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

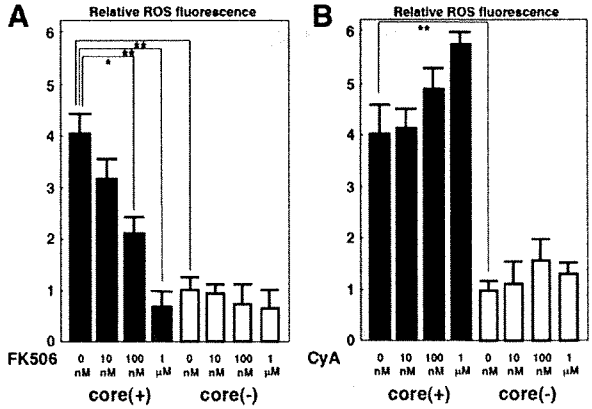

Figure 5. Effect of tacrolimus (FK506) or CyA on ROS production in HepG2 cells expressing the core protein. Results are expressed as relative brightness and normalized to control cells. **A:** Treatment with tacrolimus at 0 nmol/L, 10 nmol/L, 100 nmol/L, or 1 μmol/L. **B:** Treatment with CyA at 0 nmol/L, 10 nmol/L, 100 nmol/L, or 1 μmol/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 ± 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).

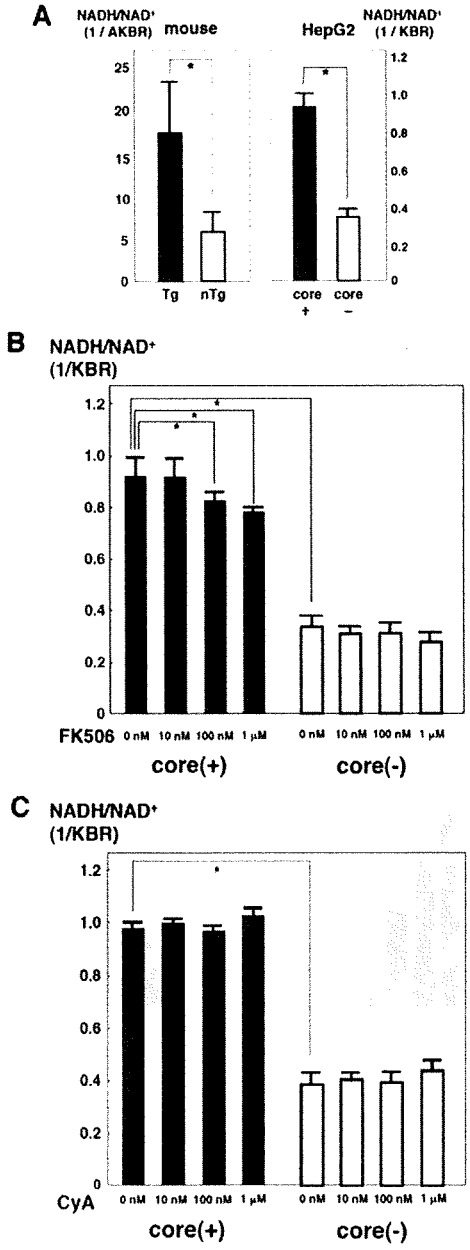

Figure 6. Effect of tacrolimus (FK506) or CyA on NADH accumulation in HepG2 cells expressing the core protein. **A:** NADH/NAD$^+$ was determined in mice (left) or HepG2 cells (right) with or without the core protein. **B:** The ketone body ratio was determined in HepG2 cells with or without the core protein after incubation with tacrolimus for 24 hours at 0 nmol/L, 10 nmol/L, 100 nmol/L, or 1 μmol/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 μmol/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 ± SE; $n = 5$ in each group. $^*P < 0.05$. AKBR, arterial KBR; Tg, transgenic mice; nTg, nontransgenic mice.

Discussion

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

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

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-

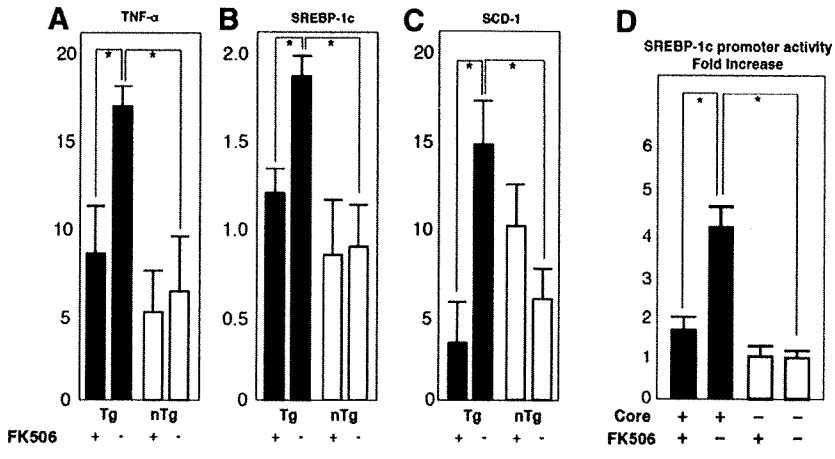

Figure 7. A–C: Effect of tacrolimus (FK506) on mRNA levels of cellular genes. The mRNA levels of TNF-α (**A**), SREBP-1c (**B**), and SCD-1 (**C**) genes were determined by real-time PCR analysis in the tacrolimus- or vehicle-treated mouse livers. The transcriptions of the genes were normalized with 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 ± 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, Craxì 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, Bréchot 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 D(−)-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 PA28γ 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. Penfornis 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

Proteomics Analysis of Mitochondrial Proteins Reveals Overexpression of a Mitochondrial Protein Chaperon, Prohibitin, in Cells Expressing Hepatitis C Virus Core Protein

Takeya Tsutsumi,[1] Mami Matsuda,[2] Hideki Aizaki,[2] Kyoji Moriya,[1] Hideyuki Miyoshi,[1] Hajime Fujie,[1] Yoshizumi Shintani,[1] Hiroshi Yotsuyanagi,[1] Tatsuo Miyamura,[2] Tetsuro Suzuki,[2] and Kazuhiko Koike[1]

The hepatitis C virus (HCV) core protein is involved in viral pathogenesis such as oxidative stress induction and lipid metabolism disturbance, and is primarily located in the cytoplasm and endoplasmic reticulum in association with lipid droplets as well as in the mitochondria. To clarify the impact of the core protein on mitochondria, we analyzed the expression pattern of mitochondrial proteins in core protein-expressing cells by two-dimensional polyacrylamide gel electrophoresis. Several proteins related to the mitochondrial respiratory chain or protein chaperons were identified by mass spectrometry. Among the identified proteins with consistently different expressions, prohibitin, a mitochondrial protein chaperon, was up-regulated not only in core-expressing cells but also in full-genomic replicon cells and livers of core-gene transgenic mice. The stability of prohibitin was increased through interaction with the core protein. Further analysis demonstrated that interaction of prohibitin with mitochondrial DNA-encoded subunits of cytochrome c oxidase (COX) was disturbed by the core protein, resulting in a significant decrease in COX activity. *Conclusion:* The HCV core protein affects the steady-state levels of a subset of mitochondrial proteins including prohibitin, which may lead to an impaired function of the mitochondrial respiratory chain with the overproduction of oxidative stress. (HEPATOLOGY 2009;50:378-386.)

The hepatitis C virus (HCV) is a causative agent of chronic hepatitis, which often leads to cirrhosis and, eventually, to the development of hepatocellular carcinoma (HCC). However, the mechanism of hepatocarcinogenesis in HCV infection is not yet fully elucidated. The HCV core protein forms the viral nucleocapsid protein and has various properties that modulate cellular processes in numerous ways. The core protein binds to cellular proteins, suppresses or enhances apoptosis, and modulates the transcription of some host genes.[1] In addition, transgenic mice expressing the core protein develop HCC,[2-4] indicating a direct contribution of the core protein to the pathogenesis of hepatitis C.

The core protein is mostly localized to the endoplasmic reticulum (ER), but we and other groups have shown its localization to the mitochondria in cultured cells and transgenic mice.[2,5,6] In addition, the double structure of mitochondrial membranes is disrupted in hepatocytes of core-gene transgenic mice.[2-4] Evidence suggests that the core protein modulates some mitochondrial functions, including fatty acid β-oxidation, the impairment of which may induce lipid abnormalities and hepatic steatosis. In addition, the mitochondrion is an important source of reactive oxygen species (ROS). In livers of transgenic

Abbreviations: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; COX, cytochrome c oxidase; ER, endoplasmic reticulum; Ero1, ER protein endoplasmic oxidoreduction-1; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HSP, heat shock protein; IFN, interferon; MnSOD, manganese superoxide dismutase; NS, nonstructural; OST48, oligosaccharylransferases-48; PDH, pyruvate dehydrogenase; PDI, protein disulfide isomerase; ROS, reactive oxygen species; TFA, trifluoroacetic acid.

From the [1]Department of Internal Medicine, Graduate School of Medicine, University of Tokyo; [2]Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan.

Received June 17, 2008; accepted March 20, 2009.

Supported by a grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science, from the Ministry of Health, Labour and Welfare of Japan (Research on Hepatitis), from the Ministry of Education, Culture, Sports, Science and Technology (Priority Area), from The Sankyo Foundation of Life Science, and from The Charitable Trust Araki Memorial Promotion Fund. T.T. is an awardee of the Research Resident Fellowship from the Viral Hepatitis Research Foundation of Japan.

Address reprint requests to: Kazuhiko Koike, M.D., Ph.D., Department of Gastroenterology, Internal Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. E-mail: kkoike-tky@umin.ac.jp; fax: (81) 3-5800-8799

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.22998

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

mice harboring the core gene, increased ROS production has been observed.[7-9] A recent study found, by the proteomic profiling of biopsy specimens, that an impairment in key mitochondrial processes, including fatty acid oxidation and oxidative phosphorylation, and in the response to oxidative stress occurs in HCV-infected human liver with advanced fibrosis.[10] Therefore, it is probable that the HCV core protein affects mitochondrial functions because such pathogenesis is observed in both HCV core-transgenic mice and HCV-infected patients.[11-13]

The recent progress in proteomics has opened new avenues for disease-related biomarker discovery. Among proteomics approaches, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is a technique for the separation and identification of proteins in a sample by displacement in two dimensions oriented at right angles to one another. This method is generally used as a component of proteomics and is the step used for the isolation of proteins for further characterization by mass spectrometry. 2D-PAGE is particularly useful when comparing two related samples such as healthy and diseased tissue. For example, proteins that are more abundant in diseased tissue may represent novel drug targets or diagnostic markers. In fact, several candidate biomarkers for many human cancers have been identified by this approach.[14] There are, however, tens of thousands of proteins in a cell, differing in abundance over six orders of magnitude. 2D-PAGE is not sensitive enough to detect rare proteins, and hence many proteins are not resolved. Therefore, splitting a sample into different fractions is often necessary to reduce the complexity of protein mixtures prior to 2D-PAGE. For this advantage, Lescuyer et al.[15] performed a 2D-PAGE of human mitochondrial proteins derived from the placenta and identified proteins mainly by peptide mass fingerprinting.

In this study, we performed a 2D-PAGE of mitochondria isolated from HepG2 cells stably expressing the HCV core protein and identified several proteins of different expressions when compared with control HepG2 cells. Among up-regulated proteins in the core-expressing cells, we focused on prohibitin, which functions as a mitochondrial protein chaperon, and found that the core protein interacts with prohibitin and represses the interaction between prohibitin and subunit proteins of cytochrome c oxidase (COX), which may lead to decreases in the expression level of the proteins and in COX activity. These results may explain the pathogenesis of liver disease in HCV infection including ROS induction.

Materials and Methods

Cells and Purification of Mitochondria. Hep39 cells,[16] which stably express the HCV core protein, and control HepG2 cells (Hepswx) were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum and 1 mg/mL G418. Mitochondria were purified using Nycodenz (Nycomed Pharma, Zürich, Switzerland) according to the protocols reported by Okado-Matsumoto et al.[17] For transient transfection experiments, HepG2 cells were transfected with a core-expression plasmid using TransIT-LT1 (Mirus Bio, Madison, WI). Huh7 cells harboring HCV genotype 1b full-genomic (RCYM1)[18] or subgenomic replicon (5-15), and livers of 3-month-old core-gene transgenic mice[2] were also used for the analysis.

2D-PAGE. Gel electrophoresis in the first dimension was performed using an immobilized pH gradient gel (Immobiline Dry Strip gel, pH 4-7 linear, 13 cm; GE Healthcare, Uppsala, Sweden). The two-dimensional separation was performed on 12.5%, 14 × 16 cm^2, SDS polyacrylamide gels. After the electrophoresis, gels were silver-stained using a silver staining kit (GE Healthcare) according to the manufacturer's protocols. The stained gels were scanned and electronic images of the gels were analyzed using ImageMaster 2D Elite software (GE Healthcare).

In-Gel Digestion and Matrix-Assisted Laser Desorption Ionization, Time-of-Flight Mass Spectrometry (MALDI-TOF-MS). Protein spots on the gels were excised and a "control" piece was cut from a blank region of the gel and processed in parallel with the sample. In-gel digestion with trypsin was performed as reported.[19] The resulting peptides were concentrated using Zip-Tip C18 (Millipore, Bedford, MA). The peptide mixtures were eluted from Zip-Tip with 75% acetonitrile in 0.1% trifluoroacetic acid (TFA). The matrix (α-cyano-4-hydroxycinnamic acid dissolved in 50% acetonitrile, 0.1% TFA) was deposited on a dried sample target. Then 0.5-μL aliquots of the analyte solution were deposited onto matrix surfaces and the solvent was allowed to evaporate at ambient temperature. The digests were analyzed with a TOF mass spectrometer, PE Biosystems Voyager DE STR MALDI (Foster City, CA).

Database Analysis. For protein identification the measured monoisotopic masses of the peptides were analyzed using MS-Fit provided by UCSF (http://prospector.ucsf.edu/ucsfhtml3.2/msfit.htm).

Immunoblotting and Immunoprecipitation. Purified mitochondria were lysed and sonicated in RIPA buffer, then centrifuged at 16,000 rpm for 10 minutes. Protein concentration was determined using a BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL). The samples were separated by sodium dodecyl sulfate (SDS)-PAGE and electrotransferred onto a polyvinylidene fluoride membrane (Immobilon; Millipore, Japan), then blocked with BlockAce (Snow Brand, To-

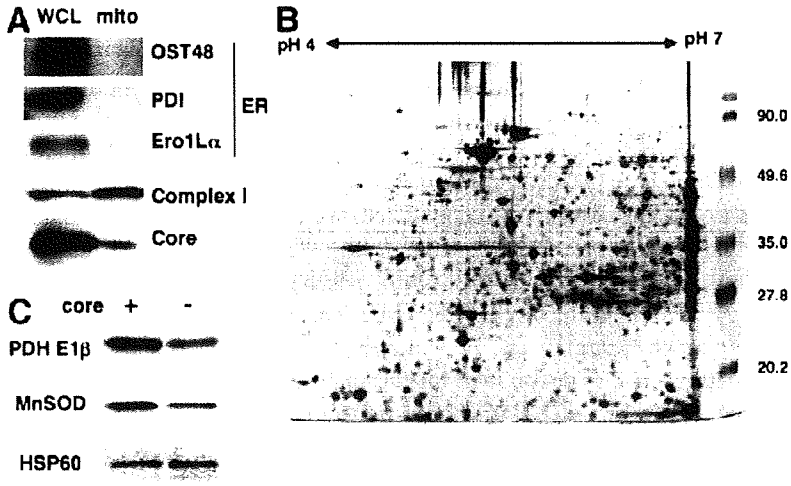

Fig. 1. 2D-PAGE of mitochondria purified from core-expressing cells. (A) Whole-cell lysates (WCL) and purified mitochondria (mito) derived from core-expressing cells were subjected to SDS-PAGE and immunoblotted with anti-core, anti-subunit of complex I (mitochondrial protein), or anti-OST48, PDI, Ero1La (ER proteins) antibodies. (B) Purified mitochondria of core-expressing cells were subjected to 2D-PAGE and the gel was stained with silver. The numbers shown on the right are molecular weights. (C) Purified mitochondria of core-expressing and control cells were subjected to SDS-PAGE and blotted with an anti-E1β subunit of PDH (PDH E1β), anti-MnSOD, or anti-HSP60 antibody.

kyo, Japan). The membrane was subsequently incubated with specific primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies and visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce). Antibodies against the core protein (Anogen, Mississauga, Canada), manganese superoxide dismutase (MnSOD) (BD Biosciences, San Jose, CA), prohibitin (Neomarkers, Fremont, CA), oligosaccharyltransferase-48 (OST48), heat shock protein (HSP) 60 (Santa-Cruz Biotechnology, Santa Cruz, CA), pyruvate dehydrogenase (PDH), ubiquinol-cytochrome c oxidoreductase, COX (Molecular Probes, Eugene, OR), protein disulfide isomerase (PDI), ER protein endoplasmic oxidoreduction-1 (Ero1)-Lα, and IκBα (Cell Signaling Technology, Danvers, MA), were used as primary antibodies. For immunoprecipitation experiments, cells were lysed in NET-N buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) and the lysates were incubated with anti-prohibitin overnight followed by the addition of protein Sepharose 4B (GE Healthcare), then washed with the same buffer five times. Immunoprecipitates were subjected to SDS-PAGE followed by immunoblotting with specific antibodies.

Determination of COX Activity. COX activity was determined with a MitoProfile Rapid Microplate Assay Kit (MitoSciences, Eugene, OR) using 10 μg of purified mitochondria. The assay was performed three times independently.

Statistical Analysis. Results are expressed as means ± SE. The significance of the difference in means was determined by Student's *t* test or Mann-Whitney's *U* test.

Results

Presence of HCV Core Protein in Purified Mitochondria. Increasing evidence suggests that the HCV core protein is localized to mitochondria as well as to ER and the nucleus. Therefore, we first investigated whether the core protein is expressed in the mitochondria of core-expressing (Hep39) cells used in this study. We used Nycodenz discontinuous gradients to extract mitochondria as described.[17] In the mitochondria derived from core-expressing HepG2 cells, the core protein was detected by immunoblotting, whereas ER resident proteins such as an ER-specific type I transmembrane protein OST48, ER-resident molecular chaperon PDI, and ER membrane-associated N-glycoprotein Ero1-Lα, were not (Fig. 1A). In this fraction, reduced nicotinamide adenine dinucleotide (NADH)-ubiquinone oxidoreductase, complex I of mitochondrial oxidative phosphorylation system, was more strongly expressed than that in the whole cell. These results indicate that the purified mitochondria fraction was free of ER, and that a portion of the core protein was localized to the mitochondria in core-expressing cells.

Proteomics Analysis of Mitochondria by 2D-PAGE. For proteomics analysis, purified mitochondrial proteins derived from core-expressing cells were subjected to 2D-PAGE followed by silver-staining of the gel. In this study we analyzed only acidic proteins using IPG strips covering pH 4 to pH 7 because the analysis of acidic proteins by 2D-PAGE is relatively easy. The mitochondrial fraction was also extracted from Hepswx, a control cell line resistant to G418 but does not express the core protein, then similarly subjected to 2D-PAGE and used for comparing the expression pattern. We repeated the above procedure (purification of mitochondria, 2D-PAGE, and silver-staining) five times, and confirmed a similar expression pattern in core-expressing cells. The representative gel image is shown in Fig. 1B. ImageMaster 2D Elite software detected about 1100 spots on the silver-stained acidic gel, i.e., at pH 4-7 and Mrs of 20-100 kDa. The number of

Table 1. Proteins of Differential Expression in Mitochondria of Core-Expressing Cells

Protein Name	Fold Change (Mean ± SD)
Increased	
Succinyl-CoA:ketoacid CoA transferase	10.43 ± 1.29
NADH-specific isocitrate dehydrogenase a subunit precursor	9.64 ± 4.66
Unknown	8.65 ± 2.40
GrpE-like protein co-chaperon	5.71 ± 0.49
Leucine aminopeptidase	4.26 ± 1.14
Pyruvate dehydrogenase E1 component b subunit	3.79 ± 1.34
CGO15alt2	3.18 ± 0.80
HSP70	3.11 ± 1.39
Prohibitin	2.60 ± 0.24
3-Hydroxyisobutyrate dehydrogenase	2.47 ± 0.77
HSPC108	2.46 ± 0.69
MnSOD	2.35 ± 0.65
Ubiquinol-cytochrome c oxidoreductase core I protein	2.00 ± 0.23
Decreased	
Aldehyde dehydrogenase 2	0.12 ± 0.02
Aldehyde dehydrogenase 5 precursor	0.25 ± 0.03
ATP synthase a subunit isoform 1	0.50 ± 0.09
Reference protein	
HSP60	1.02 ± 0.02

protein spots was smaller than those reported in a recent study investigating the human placental mitochondrial proteome.[15]

We then compared the intensity of the spots between core-expressing and control cells. Analysis of repeated experiments by Student's *t* test revealed 13 increased and three decreased spots in intensity in core-expressing cells. These spots were excised and digested with trypsin, then proteins were identified by mass spectrometry. The names of the identified proteins are listed in Table 1. Among them were proteins related to mitochondrial respiratory chain, protein chaperons, and lipid metabolism. Because antibodies to some of these proteins are commercially available, expression levels of the proteins were examined by immunoblotting. The expression levels of the PDH-E1β subunit and MnSOD, which were identified as increased proteins, were higher in core-expressing cells than in control cells (Fig. 1C), whereas that of HSP60, which was identified as having a similar expression, was unchanged.

Up-regulation of Prohibitin by the Core Protein. Among the identified proteins, we focused on prohibitin, an up-regulated protein in mitochondria of core-expressing cells (Fig. 2A). Prohibitin is a mitochondrial protein associated with cell proliferation.[20] It also works as a chaperon of mitochondrial proteins.[21,22] We confirmed an increased prohibitin expression level in core-expressing cells

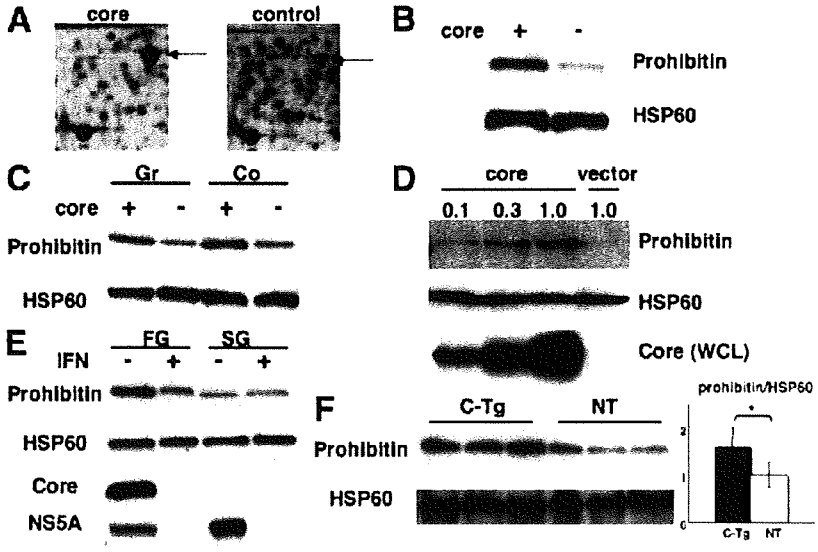

Fig. 2. Up-regulation of prohibitin in core-expressing cells. (A) Protein spot corresponding to prohibitin (arrow) in 2D-PAGE. (B) Purified mitochondria from core-expressing or control cells were subjected to SDS-PAGE and immunoblotted with anti-prohibitin or anti-HSP60 antibody. (C) Mitochondria were purified from growing (Gr) or confluent (Co) cells in 100-mm dishes and subjected to SDS-PAGE, then immunoblotted with an anti-prohibitin or anti-HSP60 antibody. (D) HepG2 cells in six-well plates were transfected with different amounts (μg) of core-expressing plasmid and mitochondrial proteins were analyzed by immunoblotting with anti-prohibitin or anti-HSP60 antibody. The expression levels of the core protein in whole-cell lysates (WCL) were also determined. (E) Cells harboring HCV replicon were untreated or treated with IFN and expression levels of prohibitin in mitochondria were determined. Expression of HCV core and NS5A proteins was also examined. FG, full-genomic replicon cells; SG, subgenomic replicon cells. (F) Expression levels of prohibitin in mitochondria were determined in liver tissues HCV core-gene transgenic and nontransgenic mice. Prohibitin/HSP60 expression levels were determined by densitometry. C-Tg, core-gene transgenic mouse; NT, nontransgenic littermate (n = 3) $^*P < 0.05$.

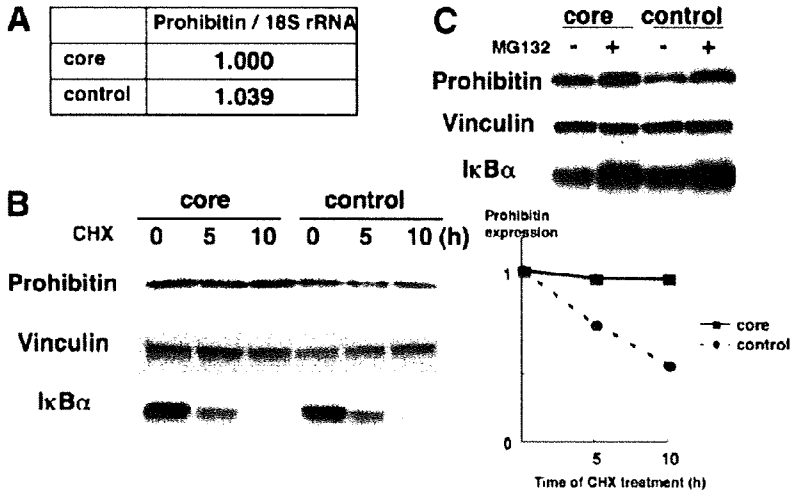

Fig. 3. Increased protein stability of prohibitin in core-expressing cells. (A) RNA was extracted from core-expressing and control cells, and the amount of specific mRNA was determined by real-time PCR with specific primers/probe against prohibitin. The amount of prohibitin mRNA was standardized by that of 18S ribosomal RNA (18S rRNA). (B) Cells were incubated with 100 ng/mL cycloheximide and harvested at the timepoints indicated above the lanes (numbers are hours of cycloheximide treatment). Whole-cell lysates were subjected to SDS-PAGE and immunoblotted with anti-prohibitin, anti-IκBα, or anti-vinculin (as an internal standard) antibody. The intensity of each band was measured by densitometry, and expression levels (prohibitin/vinculin) are shown in the right panel. (C) Cells were harvested after incubation with 20 μM MG132 for 8 hours and subjected to immunoblotting with anti-prohibitin, anti-IκBα, or anti-vinculin antibody.

by immunoblotting (Fig. 2B). Because prohibitin is associated with cell proliferation, it is possible that prohibitin expression changed according to the cell proliferative status. As shown in Fig. 2C, core-expressing cells had high prohibitin expression levels in the cells in both confluent growth and growing statuses compared with control cells. We also determined the expression levels in cells synchronized with aphidicolin followed by l-mimosine treatment and found an increased expression level in core-expressing cells (data not shown). To exclude the possibility that the increased prohibitin expression level is due to the expansion of limited cell clones, not specific to the core protein expression, we examined prohibitin expression in cells transiently expressing the core protein and found that prohibitin expression level increased dose-dependently in core-expressing cells (Fig. 2D). We also examined the prohibitin expression levels in Huh7 cells harboring full- or subgenomic HCV replicon. For this purpose, we used interferon (IFN)-treated replicon cells (cured cells) as a control. Core and nonstructural (NS)5A proteins were not detected after treatment of full-genomic replicon cells with IFN, suggesting a successful elimination of replicon. Prohibitin expression levels in cells with full-genomic replicon were increased compared with those in IFN-treated cured cells, whereas levels of prohibitin expression were low in subgenomic replicon cells regardless of IFN-treatment (Fig. 2E). In addition, prohibitin expression levels were also increased in livers of 3-month-old transgenic mice expressing the core protein compared with those in nontransgenic littermates (Fig. 2F).

We next sought to determine the mechanism of the increased steady-state level of prohibitin in core-expressing cells. To determine prohibitin messenger RNA (mRNA) expression, we performed a real-time polymerase chain reaction (PCR) using specific primers/probe. No difference in prohibitin mRNA was observed between core-expressing and control cells (Fig. 3A). We next determined the stability of prohibitin in these cells. By treating the cells with cycloheximide, the expression levels of prohibitin gradually decreased in control cells (Fig. 3B). On the other hand, in core-expressing cells prohibitin was hardly degraded by cycloheximide treatment for 10 hours, whereas IκBα was equally degraded in both cells. This result suggests that prohibitin was stabilized in the presence of the core protein. Because prohibitin has been shown to be degraded by proteasome,[23] we examined expression levels of prohibitin in the presence of proteasome inhibitor MG132. By treatment with MG132, prohibitin expression was increased to the similar level in core-expressing and control cells. These results suggest that the core protein may inhibit proteasomal degradation of prohibitin by some mechanism, including the prevention of degradation by interaction with the core protein. Then, core-expressing cells were lysed and subjected to immunoprecipitation with an anti-prohibitin antibody. As shown in Fig. 4, the core protein was coimmunoprecipitated with an anti-prohibitin antibody. To exclude a nonspecific interaction with the antibody or Sepharose beads, cells expressing a small amount of prohibitin by transfection with small interfering RNA (siRNA) against prohibitin were also examined. In these cells the amount of the coimmunoprecipitated core protein decreased. In addition, the core protein was not coimmunoprecipitated by control immunoglobulin G (IgG), indicating a specific interaction of prohibitin with the core protein. These results suggest that prohibitin expression increased in core-expressing cells owing to the increased stability presumably by interaction with the core protein.

Impaired Chaperon Function of Prohibitin in Core-Expressing Cells. We next examined the effect of

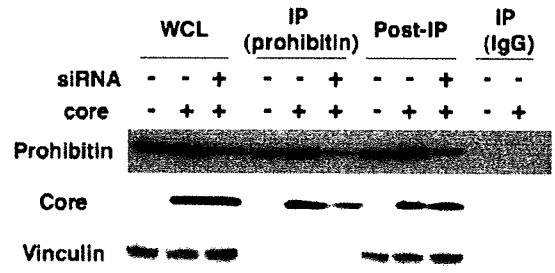

Fig. 4. Interaction of the core protein with prohibitin. Core-expressing and control cells were transfected with or without siRNA against the prohibitin gene, then harvested and lysed in NET-N buffer 3 days after transfection. Whole-cell lysates (WCL) were immunoprecipitated (IP) with an anti-prohibitin antibody or control IgG and immunoblotted with anti-prohibitin or anti-core antibody. Supernatants after the immunoprecipitation were harvested and similarly immunoblotted (Post-IP).

the interaction of prohibitin with the core protein on the function of prohibitin. Prohibitin works as a chaperon of mitochondrial proteins. Nijtmans et al.[21] demonstrated that prohibitin exerts a chaperon function particularly for the stabilization of mitochondrial DNA-encoded proteins. COX is a mitochondrial respiratory complex IV formed by 14 subunits, 10 of which are encoded by nuclear DNA and the rest by mitochondrial DNA.[24] We examined the interaction of prohibitin with subunit II of COX encoded by mitochondrial DNA. As shown in Fig. 5A, the level of COX II coimmunoprecipitated with an anti-prohibitin antibody was decreased in core-expressing cells, although the amount of immunoprecipitated prohibitin was higher than that in control cells. On the other hand, the subunit IV of COX encoded by nuclear DNA was similarly coimmunoprecipitated between core-expressing and control cells. When prohibitin expression was decreased by siRNA transfection, coimmunoprecipitation of COX subunits was similarly decreased with the amount of immunoprecipitation of prohibitin itself being low. We next determined expression levels of COX subunits in the mitochondria in these cells. Expression levels of mitochondrial DNA-encoded subunits I and II in core-expressing cells were decreased, whereas the levels of nuclear DNA-encoded subunits IV and VIb were similar to those in control cells. When transfected with prohibitin-siRNA, expression levels of all of the COX subunits examined were decreased in both core-expressing and control cells, suggesting that protein levels of these subunits are dependent on prohibitin (Fig. 5B, see Supporting Fig. 1 for densitometry). Similar data were observed when blots for COX II and IV were developed together in the same membrane (Supporting Fig. 2). We also determined COX activity in these cells and found that core-expressing cells had a significantly decreased COX activity (about 70% of that in control cells, Fig. 5C). These results suggest that interaction of prohibitin with the core protein is associated with an impaired function of prohibitin as a mitochondrial chaperon, which may trigger disordered assembly and function of mitochondrial respiratory complexes.

Discussion

In the present study we analyzed expression levels of mitochondrial proteins in HepG2 cells expressing the HCV core protein and identified a set of proteins with different expressions. Some of those proteins were related to the mitochondrial respiratory chain (Table 1). Because the core protein was shown to be associated with the induction of oxidative stress,[7-9] the core protein may modulate the expression and function of proteins forming mitochondrial respiratory complexes, which naturally

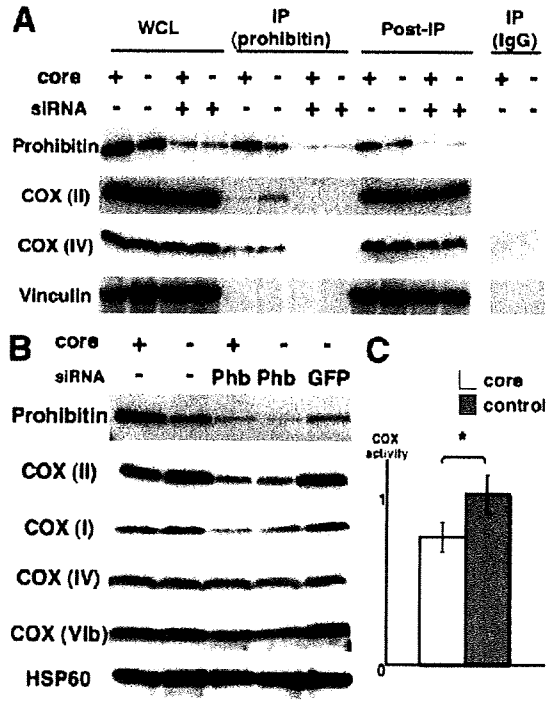

Fig. 5. Effects of core-prohibitin interaction on interaction/expression of COX subunit proteins and COX activity. (A) Whole-cell lysates (WCL) of core-expressing and control cells were subjected to immunoprecipitation with an anti-prohibitin antibody or control IgG, and the interaction of prohibitin with COX subunits was determined by immunoblotting of immunoprecipitated proteins (IP). Supernatants after the immunoprecipitation were harvested and similarly immunoblotted (Post-IP). (B) Cells were transfected with or without siRNA against the prohibitin (Phb) or GFP gene and harvested 3 days after transfection for purification of mitochondria. Purified mitochondria were subjected to SDS-PAGE and immunoblotted with several anti-COX subunits antibodies. The expression levels of HSP60 were also examined as an internal control. (C) COX activity was determined by measuring cytochrome c oxidation. The activity was normalized by taking the average rate of control cells as 1. Data shown are means ± SE (n = 5). *$P < 0.05$.

leads to ROS accumulation. In addition, MnSOD, which plays a key role in protecting cells from oxidative damage, was up-regulated in core-expressing cells, reflecting ROS increase in the cells. Several protein chaperons such as HSP70 and GrpE-like protein co-chaperon were also identified as up-regulated proteins. Because these proteins are known to be important in the mitochondrial protein-import mechanisms, the modulated expression of these proteins may be associated with the different expressions of the identified mitochondrial proteins.

Prohibitin, a mitochondrial protein chaperon, was identified as an up-regulated protein in core-expressing cells. Prohibitin is a ubiquitously expressed and highly conserved protein that was originally determined to play a predominant role in inhibiting cell-cycle progression and cellular proliferation by attenuating DNA synthesis.[20,25] Prohibitin is present in the nucleus and interacts with transcription factors that are important in cell cycle progression. In core-expressing cells used in this study, prohibitin was also detected in the nucleus and its expression level was also higher than that in control Hepswx cells or HepG2 cells (data not shown). The growth rate of core-expressing cells, however, was similar to that of control cells (data not shown). The physiological significance of the high expression level of prohibitin in the nucleus remains to be determined, but it may be related to enhanced apoptosis by Fas ligand, as shown by Ruggieri et al.,[16] because prohibitin interacts with E2F, Rb, and p53 and modulates the transcription activity of these factors and induces apoptosis.[26,27]

Mitochondrial prohibitin acts as a protein chaperon by stabilizing newly synthesized mitochondrial translation products through direct interaction.[21] We examined the interaction between prohibitin and mitochondrially encoded subunit II of COX and found a suppressed interaction between these proteins in core-expressing cells. In addition, there are several studies that showed the association of prohibitin with the assembly of mitochondrial respiratory complex I as well as complex IV (COX).[21,28] Complex I also consists of both nuclear- and mitochondrial-DNA-encoded subunits; therefore, it is probable that the assembly and function of complex I are impaired by the core protein. We attempted to examine the interaction of prohibitin with the mitochondrial DNA-encoded subunit of complex I, but commercially available antibodies against this subunit could not detect the protein itself by immunoblotting (data not shown). With respect to the complex I function, we found a decreased complex I activity in core-expressing cells (H. Miyoshi et al., manuscript in preparation). Other groups have also shown that complex I activity is decreased in the liver of transgenic mice harboring HCV core and envelope genes[9] as well as in cultured cells.[29] From these findings, the interaction between prohibitin and the core protein may impair the function of complex I as well as complex IV, leading to an increase in ROS production. In fact, the suppression of the prohibitin function is shown to result in an increased production of ROS,[30] a phenomenon observed in core-expressing cells used in this study (Miyoshi et al., in prep.) as well as in the liver of core-gene transgenic mice.[7,8] Interestingly, Berger and Yaffe[31] showed that loss of function of prohibitin leads to an altered mitochondrial morphology, that is, the loss of the normal reticular morphology and organized mitochondrial distribution. In hepatocytes from the core-gene transgenic mice, we observed a change in morphology of mitochondria, a disappearance of the double structure of mitochondrial membranes.[2] These changes in mitochondrial morphology are somewhat different, but the dysfunction of prohibitin may be responsible for the morphological abnormality of mitochondria observed in the core-gene transgenic mice.

We concluded that prohibitin overexpression is due to increased stability induced by the interaction with the core protein. In this study we showed that prohibitin might be degraded by proteasome, although we could not detect ubiquitinated forms of prohibitin. If the degradation is mediated by ubiquitin as reported,[23] it is possible that the interaction with the core protein interferes with ubiquitin-binding and protects prohibitin from degradation by proteasome. Some posttranslational protein modifications such as phosphorylation are other possible factors for the stabilization, because prohibitin can be serine-phosphorylated[32]; however, in our examination no serine/threonine/tyrosine phosphorylation of prohibitin was detected in core-expressing cells (data not shown). Thus far, there are no studies showing that prohibitin stabilization leads to a suppressed function as a mitochondrial chaperon. Therefore, this finding is novel and noteworthy because the prohibitin expression level has been considered to be proportional to the chaperon function. Prohibitin is highly expressed in several human tumors.[33,34] In addition, a 2D-PAGE of the hepatoma cell line HCC-M identified prohibitin as a positively regulated protein.[35] In these studies, the mechanism of prohibitin overexpression was not elucidated, but considering that prohibitin is associated with the inhibition of cell proliferation, the function of prohibitin is suppressed by stabilization by some molecules in the tumor, similar to the mechanism we suggest in the current study.

In addition to HepG2 cells constitutively expressing the core protein, increased prohibitin expression levels were also found in livers of core-gene transgenic mice.

The difference in expression levels between the transgenic mice and nontransgenic littermates, however, was a little bit smaller than that in the studies of HepG2 cells. This may be due to the low expression level of the core protein in the transgenic mice compared with that in core-expressing HepG2 cells because the expression level of prohibitin was proportionally increased to that of the core protein as shown in this study (Fig. 2D). Otherwise, there might be some *in vivo* mechanism for suppressing prohibitin expression in mice.

In this study, COX subunit IV as well as II were found to interact with prohibitin (Fig. 5A). Although there are no studies demonstrating that prohibitin also works as chaperon for nuclear DNA-encoded mitochondrial proteins as far as we investigated, knockdown of prohibitin expression by siRNA led to decreases in expression levels of both nuclear (COX IV, VIb) and mitochondrial (COX I, II) DNA-encoded subunits in mitochondria (Fig. 5B and Supporting Figs. 1 and 2). We showed that COX IV interacts with prohibitin (Fig. 4), suggesting that prohibitin also works for stable expression of nuclear DNA-encoded COX IV. Degrees of decrease in COX IV and VIb expression, however, were smaller than those in I and II. Prohibitin might contribute to stabilization of COX IV and VIb by mechanism(s) other than chaperon function. Steglich et al.[36] showed that prohibitin regulates protein degradation by the m-AAA protease in mitochondria. Recently, Da Cruz et al.[37] showed that SLP-2, a member of the stomatin gene family, interacts with prohibitin and regulates the expression of mitochondrial proteins such as COX IV and ND6 of complex I encoded by nuclear DNA by AAA proteases. In view of these findings, COX IV and VIb expression in mitochondria is dependent on prohibitin but other factors may also be involved in the attainment of stable expression of these subunits. The expression levels of COX II and IV in the whole-cell lysates were not so drastic among cell samples (Fig. 5A) compared to those in the mitochondria (Fig. 5B). The reason is not clear, but it is possible that redundant proteins such as improperly folded proteins by lack of chaperons were included in the whole-cell lysates.

In summary, we analyzed mitochondrial proteins in core-expressing HepG2 cells by proteomics analysis and identified prohibitin as an up-regulated protein. The dysfunction of prohibitin induced by the core protein may lead to ROS overproduction in the mitochondrion, which plays a key role in the pathogenesis of chronic hepatitis C. The restoration of prohibitin function might be a therapeutic option for correcting the dysregulated assembly and dysfunction of mitochondrial respiratory chain complexes.

Acknowledgment: We thank S. Shinzawa, M. Yahata, and S. Yoshizaki for technical assistance.

References

1. Suzuki R, Suzuki T, Ishii K, Matsuura Y, Miyamura T. Processing and functions of Hepatitis C virus proteins. Intervirology 1999;42:145-152.
2. Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat Med 1998;4:1065-1067.
3. Naas T, Ghorbani M, Alvarez-Maya I, Lapner M, Kothary R, De Repentigny Y, et al. Characterization of liver histopathology in a transgenic mouse model expressing genotype 1a hepatitis C virus core and envelope proteins 1 and 2. J Gen Virol 2005;86:2185-2196.
4. Machida K, Cheng KT, Lai CK, Jeng KS, Sung VM, Lai MM. Hepatitis C virus triggers mitochondrial permeability transition with production of reactive oxygen species, leading to DNA damage and STAT3 activation. J Virol 2006;80:7199-7207.
5. Suzuki R, Sakamoto S, Tsutsumi T, Rikimaru A, Tanaka K, Shimoike T, et al. Molecular determinants for subcellular localization of hepatitis C virus core protein. J Virol 2005;79:1271-1281.
6. Schwer B, Ren S, Pietschmann T, Kartenbeck J, Kaehlcke K, Bartenschlager R, et al. Targeting of hepatitis C virus core protein to mitochondria through a novel C-terminal localization motif. J Virol 2004;78:7958-7968.
7. Moriya K, Nakagawa K, Santa T, Shintani Y, Fujie H, Miyoshi H, et al. Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocarcinogenesis. Cancer Res 2001;61:4365-4370.
8. Okuda M, Li K, Beard MR, Showalter LA, Scholle F, Lemon SM, et al. Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. Gastroenterology 2002;122:366-375.
9. Korenaga M, Wang T, Li Y, Showalter LA, Chan T, Sun J, et al. Hepatitis C virus core protein inhibits mitochondrial electron transport and increases reactive oxygen species (ROS) production. J Biol Chem 2005;280:37481-37488.
10. Diamond DL, Jacobs JM, Paeper B, Proll SC, Gritsenko MA, Carithers RL Jr, et al. Proteomic profiling of human liver biopsies: hepatitis C virus-induced fibrosis and mitochondrial dysfunction. HEPATOLOGY 2007;46:649-657.
11. Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Ishibashi K, Matsuura Y, et al. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. J Gen Virol 1997;78(Pt 7):1527-1531.
12. Moriya K, Todoroki T, Tsutsumi T, Fujie H, Shintani Y, Miyoshi H, et al. Increase in the concentration of carbon 18 monounsaturated fatty acids in the liver with hepatitis C: analysis in transgenic mice and humans. Biochem Biophys Res Commun 2001;281:1207-1212.
13. Fujie H, Yotsuyanagi H, Moriya K, Shintani Y, Tsutsumi T, Takayama T, et al. Steatosis and intrahepatic hepatitis C virus in chronic hepatitis. J Med Virol 1999;59:141-145.
14. Cho WC. Contribution of oncoproteomics to cancer biomarker discovery. Mol Cancer 2007;6:25.
15. Lescuyer P, Strub JM, Luche S, Diemer H, Martinez P, Van Dorsselaer A, et al. Progress in the definition of a reference human mitochondrial proteome. Proteomics 2003;3:157-167.
16. Ruggieri A, Harada T, Matsuura Y, Miyamura T. Sensitization to Fas-mediated apoptosis by hepatitis C virus core protein. Virology 1997;229:68-76.
17. Okado-Matsumoto A, Fridovich I. Subcellular distribution of superoxide dismutases (SOD) in rat liver: Cu,Zn-SOD in mitochondria. J Biol Chem 2001;276:38388-38393.
18. Murakami K, Ishii K, Ishihara Y, Yoshizaki S, Tanaka K, Gotoh Y, et al. Production of infectious hepatitis C virus particles in three-dimensional cultures of the cell line carrying the genome-length dicistronic viral RNA of genotype 1b. Virology 2006;351:381-392.

19. Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 1996;68: 850-858.
20. Mishra S, Murphy LC, Murphy LJ. The prohibitins: emerging roles in diverse functions. J Cell Mol Med 2006;10:353-363.
21. Nijtmans LG, de Jong L, Artal Sanz M, Coates PJ, Berden JA, Back JW, et al. Prohibitins act as a membrane-bound chaperone for the stabilization of mitochondrial proteins. EMBO J 2000;19:2444-2451.
22. Back JW, Sanz MA, De Jong L, De Koning LJ, Nijtmans LG, De Koster CG, et al. A structure for the yeast prohibitin complex: structure prediction and evidence from chemical crosslinking and mass spectrometry. Protein Sci 2002;11:2471-2478.
23. Thompson WE, Ramalho-Santos J, Sutovsky P. Ubiquitination of prohibitin in mammalian sperm mitochondria: possible roles in the regulation of mitochondrial inheritance and sperm quality control. Biol Reprod 2003; 69:254-260.
24. Fontanesi F, Soto IC, Horn D, Barrientos A. Assembly of mitochondrial cytochrome c-oxidase, a complicated and highly regulated cellular process. Am J Physiol Cell Physiol 2006;291:C1129-C1147.
25. Mishra S, Murphy LC, Nyomba BL, Murphy LJ. Prohibitin: a potential target for new therapeutics. Trends Mol Med 2005;11:192-197.
26. Fusaro G, Dasgupta P, Rastogi S, Joshi B, Chellappan S. Prohibitin induces the transcriptional activity of p53 and is exported from the nucleus upon apoptotic signaling. J Biol Chem 2003;278:47853-47861.
27. Joshi B, Ko D, Ordonez-Ercan D, Chellappan SP. A putative coiled-coil domain of prohibitin is sufficient to repress E2F1-mediated transcription and induce apoptosis. Biochem Biophys Res Commun 2003;312:459-466.
28. Bourges I, Ramus C, Mousson de Camaret B, Beugnot R, Remacle C, Cardol P, et al. Structural organization of mitochondrial human complex I: role of the ND4 and ND5 mitochondria-encoded subunits and interaction with prohibitin. Biochem J 2004;383:491-499.
29. Piccoli C, Scrima R, Quarato G, D'Aprile A, Ripoli M, Lecce L, et al. Hepatitis C virus protein expression causes calcium-mediated mitochondrial bioenergetic dysfunction and nitro-oxidative stress. HEPATOLOGY 2007;46:58-65.
30. Theiss AL, Idell RD, Srinivasan S, Klapproth JM, Jones DP, Merlin D, et al. Prohibitin protects against oxidative stress in intestinal epithelial cells. FASEB J 2007;21:197-206.
31. Berger KH, Yaffe MP. Prohibitin family members interact genetically with mitochondrial inheritance components in Saccharomyces cerevisiae. Mol Cell Biol 1998;18:4043-4052.
32. Ross JA, Nagy ZS, Kirken RA. The PHB1/2 phosphocomplex is required for mitochondrial homeostasis and survival of human T cells. J Biol Chem 2008;283:4699-4713.
33. Coates PJ, Nenutil R, McGregor A, Picksley SM, Crouch DH, Hall PA, et al. Mammalian prohibitin proteins respond to mitochondrial stress and decrease during cellular senescence. Exp Cell Res 2001;265:262-273.
34. Asamoto M, Cohen SM. Prohibitin gene is overexpressed but not mutated in rat bladder carcinomas and cell lines. Cancer Lett 1994;83:201-207.
35. Seow TK, Ong SE, Liang RC, Ren EC, Chan L, Ou K, et al. Two-dimensional electrophoresis map of the human hepatocellular carcinoma cell line, HCC-M, and identification of the separated proteins by mass spectrometry. Electrophoresis 2000;21:1787-1813.
36. Steglich G, Neupert W, Langer T. Prohibitins regulate membrane protein degradation by the m-AAA protease in mitochondria. Mol Cell Biol 1999; 19:3435-3442.
37. Da Cruz S, Parone PA, Gonzalo P, Bienvenut WV, Tondera D, Jourdain A, et al. SLP-2 interacts with prohibitins in the mitochondrial inner membrane and contributes to their stability. Biochim Biophys Acta 2008;1783: 904-911.

Hepatology Research 2009; 39: 657–663 doi: 10.1111/j.1872-034X.2009.00517.x

Original Article

Chronic hepatitis C in patients co-infected with human immunodeficiency virus in Japan: a retrospective multicenter analysis

Hiroshi Yotsuyanagi,[1] Yoshimi Kikuchi,[2] Kunihisa Tsukada,[1,2] Kyouji Nishida,[3] Michio Kato,[4] Hironori Sakai,[5] Junki Takamatsu,[6] Shuhei Hige,[7] Kazuaki Chayama,[8] Kyoji Moriya[1] and Kazuhiko Koike[1]

[1]*Department of Internal Medicine, Graduate School of Medicine, University of Tokyo,* [2]*AIDS Clinical Center, International Medical Center of Japan,* [3]*Department of Laboratory Medicine, Tokyo Medical University, Tokyo,* [4]*Department of Gastroenterology, Osaka National Hospital, Osaka,* [5]*Department of Gastroenterology, Kyushu National Hospital, Fukuoka,* [6]*Division of Transfusion Medicine, Nagoya University Hospital, Nagoya,* [7]*Department of Gastroenterology and Hematology, Hokkaido University Graduate School of Medicine, Sapporo and* [8]*Department of Medicine and Molecular Science, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan*

Aim: A nationwide survey in Japan revealed that nearly one-fifth of human immunodeficiency virus (HIV)-positive patients are co-infected with hepatitis C virus (HCV). We conducted a study to further analyze the features of liver disease in HIV–HCV co-infected patients.

Methods: We analyzed 297 patients from eight hospitals belonging to the HIV/AIDS Network of Japan.

Results: HCV genotypes 1, 2, 3, 4 and mixed genotypes were detected in 55.2, 13.7, 18.9, 0.9 and 11.3% of patients, respectively, in contrast to the fact that only genotypes 1 and 2 are detected in HCV mono-infected patients in Japan. This is compatible with the transmission of HCV through imported blood products contaminated by HCV. Sixteen of 297 HIV–HCV co-infected patients had advanced liver disease accompanied by ascites, hepatic encephalopathy or hepatocellular carcinoma. The average age of such patients was 41.1 ± 14.0 years, which was much younger than that of HCV mono-infected patients with the same complications. The progression speed of liver disease estimated from the changes in the levels of serum albumin, bilirubin, or platelet was slower in patients who achieved sustained virological response with interferon treatment than in those who did not receive it. The overall sustained virological response rate to interferon treatment was 43.3%.

Conclusions: Our findings suggest that liver disease is more advanced in HIV–HCV co-infected patients than in HCV mono-infected patients, and interferon treatment may retard the progression of liver disease in such patients.

Key words: acquired immunodeficiency syndrome, chronic liver disease, genotype, interferon therapy

INTRODUCTION

THE PROGNOSIS OF human immunodeficiency virus (HIV) infection has markedly improved since the introduction of hyperactive anti-retroviral therapy (HAART).[1,2] Opportunistic infection has been prevented or properly managed, resulting in lower mortality rates. Liver disease, in particular related to hepatitis C virus (HCV) infection, has now become the main cause of mortality among HIV-infected patients on HAART in Western countries.[3,4] A national survey among Japanese HIV-infected patients with coagulation disorders has shown that the mortality rate related to HCV-related liver disease after 1997 was twofold that before 1997.[5] In Japan, therefore, HCV infection may also be a major cause of death in HIV–HCV co-infected patients. However, there has been no extensive analysis of liver disease in HIV–HCV co-infected patients in Japan.

Correspondence: Professor Kazuhiko Koike, Department of Infectious Diseases, Internal Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Email: kkoike-tky@umin.ac.jp

Received 20 January 2009; revised 9 February 2009; accepted 10 February 2009.