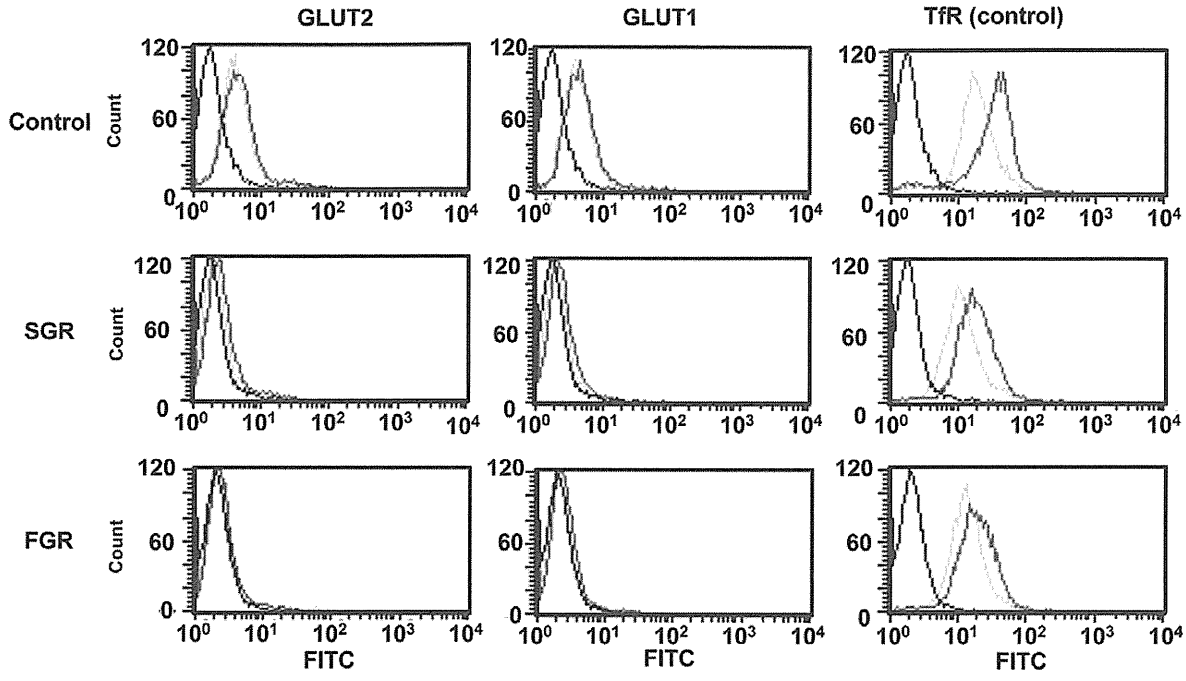

Fig. 5. Effects of lactacystin treatment on cell surface expression of GLUT2, GLUT1 and transferrin receptor (TfR). Cells were treated with lactacystin (10 μM) overnight to inhibit proteasomal degradation, and analyzed by flow cytometry. Cells treated with lactacystin are shown in red line and those left untreated in blue line. The negative controls stained with FITC-conjugated antibody alone are shown in black line.

of GLUT1 and GLUT2 expression is primarily involved in the decreased glucose uptake in SGR, FGR and HCV-infected cells.

3.8. Decreased GLUT2 expression in hepatocytes obtained from HCV-infected patients

GLUT2 is the principal glucose transporter expressed in hepatocytes *in vivo*. As shown in Fig. 7B, practically all hepatocytes obtained from patients without HCV infection showed positive staining for GLUT2, which was most evidently observed near the plasma membrane. On the other hand, hepatocytes obtained from HCV-infected patients showed markedly reduced GLUT2 staining in most, if not the entire, areas of the section, compared with the uninfected control (Fig. 7D). This heterogeneous staining pattern might reflect concomitant presence of areas comprising either virus-infected or uninfected hepatocytes in a tissue sample. Whereas all the sections obtained from 8 patients without HCV infection showed evenly positive staining for GLUT2, sections from 8 (89%) of 9 HCV-infected patients showed moderately to markedly reduced GLUT2 staining (Table 2). Reduced GLUT2 staining was observed also with hepatocytes in the liver tissues obtained from HBV-infected patients. However, the areas of reduced GLUT2 staining appeared to be more restricted in sections obtained from HBV-infected patients than in those from HCV-infected ones.

4. Discussion

HCV infection is known as an initiation and precipitating factor of type 2 diabetes [7–10,26,27]. Progression of liver fibrosis induced by persistent viral infection may induce diabetes [28]. Furthermore, it has been reported that the prevalence of diabetes is higher among patients with HCV-associated liver cirrhosis than in those with HBV-associated cirrhosis [7]. It is likely, therefore, that HCV infection itself is a risk factor of diabetes. Previous reports suggest that HCV infection directly causes insulin resistance that would cause the progression of diabetes [29–31]. However, the underlying mechanism(s) is not yet completely elucidated. In this study, we analyzed the effect of HCV infection on cellular glucose uptake and expression of glucose transporters.

We observed that glucose uptake was suppressed in cells harboring HCV RNA replicons (SGR and FGR) and those infected with HCV than in the control cells (Fig. 3). It has been reported that glucose disposal *in vivo* occurs through both insulin-dependent and insulin-independent mechanism [32]. We observed that treatment of SGR, FGR and the control Huh-7.5 cells with insulin (10^{-4} M to 10^{-9} M) increased glucose uptake by only about 50% from their basal levels (data not shown). Nevertheless, decreased glucose uptake by HCV-infected hepatocytes is a potential cause of hyperglycemia as the liver is a big organ accounting for 2% of the total body weight.

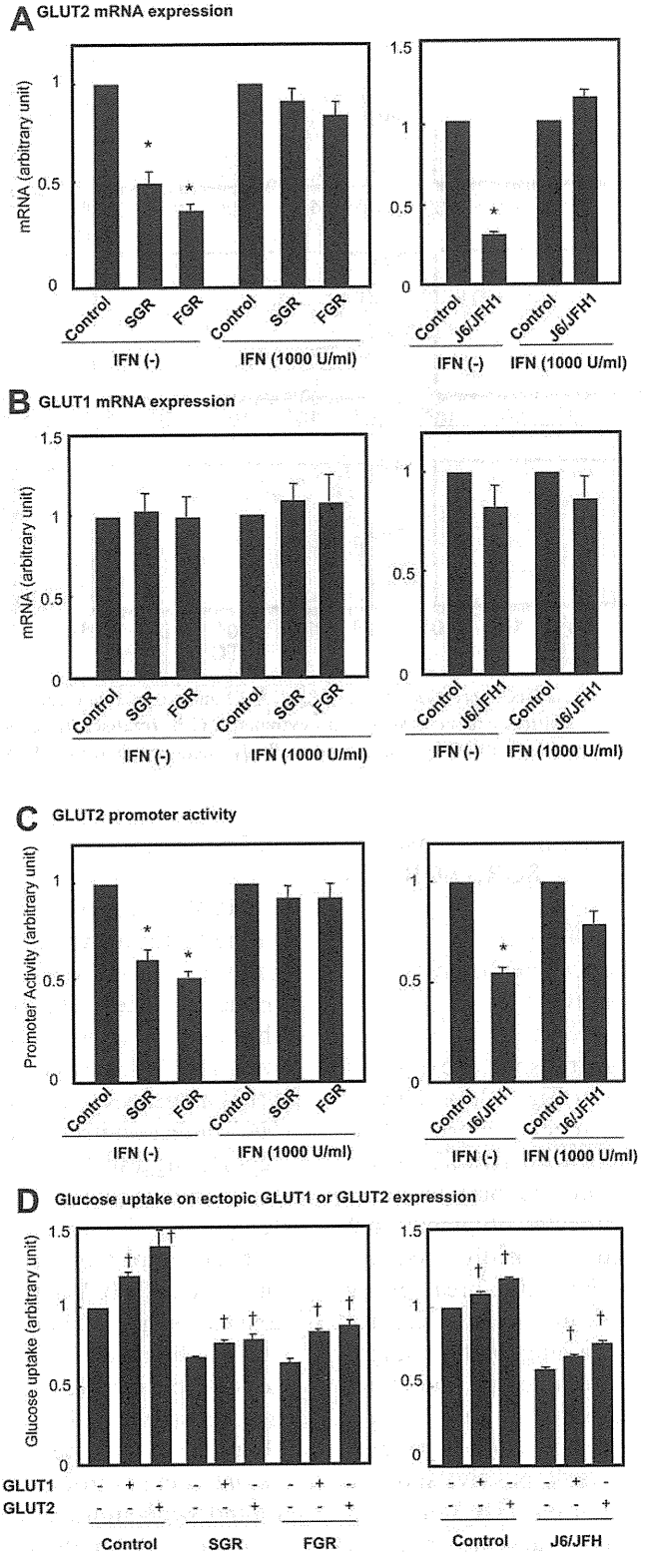

Fig. 6. Differential suppression of GLUT2 and GLUT1 mRNAs by HCV replication. (A and B) Quantitative RT-PCR analysis of mRNA for GLUT2 (A) and GLUT1 (B). mRNA expression levels of GLUT2 and GLUT1 in SGR, FGR and HCV-infected cells were determined and normalized with β-glucuronidase mRNA levels. In parallel, cells were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to quantitative RT-PCR analysis. Data represent mean ± SEM of three independent experiments. $^{*}P < 0.01$, compared with the control. (C) GLUT2 promoter activities in SGR and FGR, HCV-infected cells were analyzed using luciferase reporter assay. In parallel, cells were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to luciferase reporter assay. Data represent mean ± SEM of five independent experiments. $^{*}P < 0.01$, compared with the control. (D) Glucose uptake in cells ectopically expressing GLUT1 or GLUT2. Data represent mean ± SEM of two independent experiments. $^{\dagger}P < 0.01$, compared with mock transfected control.

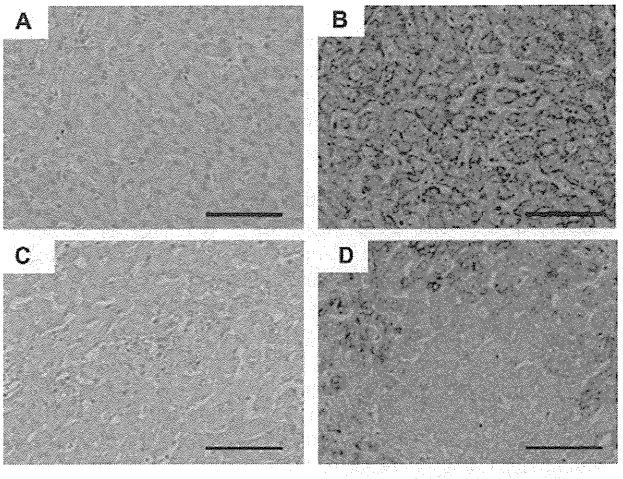

Fig. 7. Down-regulation of GLUT2 expression in HCV-infected human liver tissues *in vivo*. Normal human adult liver tissues (A and B) and HCV-infected, non-cancerous liver tissues (C and D) were fixed with formalin, sectioned and stained with normal rabbit IgG (A and C) or polyclonal anti-GLUT2 antibody (B and D). Scale bar = 100 μm.

Any proliferating cell requires energy sources, including glucose, and GLUTs play an important role in glucose uptake into the cell. In the liver, GLUT2 is the predominant glucose transporter, which regulates glucose metabolism by mediating a bidirectional transport, both entry and exit, of glucose into and from hepatocytes [13]. GLUT1, on the other hand, is known to be expressed in malignant cells including hepatocellular carcinoma [12,13] and a wide variety of cultured cells. In the present study we found that cell surface expression of GLUT2 and GLUT1 was markedly suppressed in SGR, FGR and HCV-infected cells compared to the control (Fig. 4A and B).

Table 2
Reduction of GLUT2 expression in hepatocytes of HCV-infected and HBV-infected human liver tissues.

Liver tissues	Sample No.	Reduction of GLUT2 expression
Uninfected	1	– *
	2	–
	3	–
	4	–
	5	–
	6	–
	7	–
	8	–
HCV-infected	9	1+ (Focal)[a]
	10	1+ (Focal)
	11	3+ (Diffuse)
	12	3+ (Diffuse)
	13	3+ (Diffuse)
	14	3+ (Focal)
	15	–
	16	2+ (Focal)
	17	3+ (Diffuse)
HBV-infected	18	–
	19	3+ (Diffuse)
	20	1+ (Focal)
	21	–
	22	2+ (Focal)
	23	1+ (Focal)
	24	2+ (Focal)

* -, no reduction; 1+, weak reduction; 2+, moderate reduction; 3+, strong reduction.

[a] Parentheses indicate either focal or diffuse appearance of the areas with reduced GLUT2 expression in each liver tissue sample.

GLUT2 expression is regulated at the transcriptional level, at least partly, by glucose [33]. It has been reported that hyperglycemia increases the GLUT2 mRNA and protein expression in an *in vivo* study [34]. Our present study demonstrated that GLUT2 mRNA expression was significantly suppressed in SGR, FGR and HCV-infected cells compared to the control (Fig. 6A). Consistent with this result, GLUT2 promoter activities, as measured by luciferase reporter assay, were suppressed in SGR, FGR and HCV-infected cells (Fig. 6C). In this connection, it was reported that GLUT2 promoter activities were up-regulated by sterol response element-binding protein (SREBP)-1c [35,36]. We confirmed in our study that GLUT2 promoter activities were up-regulated by over-expression of human SREBP-1c, and that the SREBP-1c-mediated GLUT2 promoter activities were suppressed significantly in SGR, FGR and HCV-infected cells (data not shown).

Unlike GLUT2 mRNA, GLUT1 mRNA was not suppressed by HCV RNA replication or HCV infection (Fig. 6B). Nevertheless, cell surface expression of GLUT1 was markedly down-regulated in SGR and FGR cells (Fig. 4A). As GLUT1 surface expression was not restored by treatment with lactacystin, a potent proteasome inhibitor (Fig. 5), it was unlikely that HCV-mediated suppression of GLUT1 surface expression was mediated through increased degradation by the ubiquitin-proteasome system. We assume that intracellular trafficking of GLUT1 (and possibly GLUT2 as well) is impaired by HCV RNA replication although we could not precisely prove it due mainly to the lack of an appropriate antibody that enables us to monitor GLUT1 trafficking. Further study is needed to elucidate the issue.

By means of immunohistochemical analysis, we confirmed that GLUT2 was strongly expressed in hepatocytes of the liver tissues obtained from all of 8 individuals without HCV infection (Fig. 7B and Table 2). More importantly, we demonstrated that GLUT2 expression was significantly down-regulated in hepatocytes obtained from 8 of 9 HCV-infected patients (Fig. 7D and Table 2). Interestingly, the areas where GLUT2 down-regulation was observed appeared to be scattered across the liver tissue sections. This may reflect the general observation that a group of hepatocytes in limited areas of the hepatic lobules, but not all the hepatocytes, are infected with HCV *in vivo*. By means of real-time quantitative PCR analysis, we found a tendency that levels of GLUT2 mRNA expression in liver tissues obtained from HCV-infected patients were lower than that obtained from uninfected controls although the dif-

ference was not statistically significant (data not shown). As stated above, not all the hepatocytes in the liver were infected with HCV and, therefore, the possible reduction of GLUT2 mRNA expression in HCV-infected hepatocytes might have been masked by the normal levels of expression in uninfected hepatocytes concomitantly present in the same tissue samples.

It should also be noted that GLUT2 staining was also reduced in hepatocytes obtained from HBV-infected patients, though to a lesser extent than that from HCV-infected ones (Table 2). We assume that inflammatory responses in the liver may trigger some intracellular event that leads to decreased GLUT2 expression in hepatocytes *in vivo*.

In conclusion, we have demonstrated for the first time that HCV replication inhibits cellular glucose uptake through down-regulation of cell surface expression of GLUT2 and possibly GLUT1. It is conceivable that the decreased glucose uptake by hepatocytes causes impaired glucose metabolism, leading eventually to the initiation and progression of diabetes mellitus during a prolonged period of HCV persistence.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2008.12.029.

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Research Article

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Pathogenesis of lipid metabolism disorder in hepatitis C: Polyunsaturated fatty acids counteract lipid alterations induced by the core protein

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Background & Aims: Disturbance in lipid metabolism is one of the features of chronic hepatitis C, being a crucial determinant of the progression of liver fibrosis. Experimental studies have revealed that the core protein of hepatitis C virus (HCV) induces steatosis.

Methods: The activities of fatty acid metabolizing enzymes were determined by analyzing the fatty acid compositions in HepG2 cells with or without core protein expression.

Results: There was a marked accumulation of triglycerides in core-expressing HepG2 cells. While the oleic/stearic acid (18:1/18:0) and palmitoleic/palmitic acid ratio (16:1/16:0) were comparable in both the core-expressing and the control cells, there was a marked accumulation of downstream product, 5,8,11-eicosatrienoic acid (20:3(n-9)) in the core-expressing HepG2 cells. The addition of eicosatetraynoic acid, which inhibits delta-6 desaturase activity which is inherently high in HepG2 cells, led to a marked accumulation of oleic and palmitoleic acids in the core-expressing cells, showing that delta-9 desaturase was activated by the core protein. Eicosapentaenoic acid (20:5(n-3)) or arachidonic acid (20:4(n-6)) administration significantly decreased delta-9 desaturase activity, the concentration of 20:3(n-9), and triglyceride accumulation. This lipid metabolism disorder was associated with NADH accumulation due to mitochondrial dysfunction, and was reversed by the addition of pyruvate through NADH utilization.

Conclusions: The fatty acid enzyme, delta-9 desaturase, was activated by HCV core protein and polyunsaturated fatty acids counteracted this impact of the core protein on lipid metabolism. These results may open up new insights into the mechanism of lipid metabolism disorder associated with HCV infection and provide clues for the development of new therapeutic devices.

Keywords: Steatosis; Oleic acid; Core protein; Lipid metabolism; Desaturase; Hepatocellular carcinoma; NADH.

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Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; PUFA, polyunsaturated fatty acids; PPAR, peroxisome proliferators-activated receptors; SREBP, sterol regulatory element binding protein; EPA, eicosapentaenoic acid; AA, arachidonic acid; ETYA, eicosatetraynoic acid; NADH, nicotinamide adenine dinucleotide; KBR, ketone body ratio.

Introduction

Persistent hepatitis C virus (HCV) infection leads to the development of chronic hepatitis, cirrhosis, and eventually, hepatocellular carcinoma (HCC), thereby being a serious problem worldwide both in medical and in socio-economical settings [1]. Histologically, several distinct features, such as bile duct damage, lymphoid follicle formation, and steatosis, (fatty change) characterize chronic hepatitis C [2–4]. Among these, steatosis is reproducible in experimental systems, both *in vitro* and *in vivo*, in which HCV proteins, particularly the core protein of HCV, are expressed. The introduced core gene induces the formation of lipid droplets in the cytoplasm of cultured cells [5,6], and in transgenic mice, it induces hepatic steatosis resembling that in chronic hepatitis C patients [7–10].

In addition, evidence has accumulated showing that steatosis is a crucial determining factor for the progression of liver fibrosis [11–13]. Steatosis and serum lipid profiles are also associated with sustained virological response to ribavirin/interferon combination therapy [14,15]. Moreover, HCV transgenic mice resemble chronic hepatitis C patients in terms of the development of HCC, implying that the HCV core protein is one of the most important viral molecules in the pathogenesis of hepatitis C [16,17]. It would thus be meaningful to explore the precise role of the core protein in modulating lipid metabolism, which may also be involved in hepatocarcinogenesis. More recently, involvement of the metabolism of lipids such as sphingolipids or cholesterol has been implicated in the replication of HCV, with a formation of lipid rafts, which are considered to be the place for HCV replication [18,19], hereby highlighting again the importance of lipid metabolism in HCV infection.

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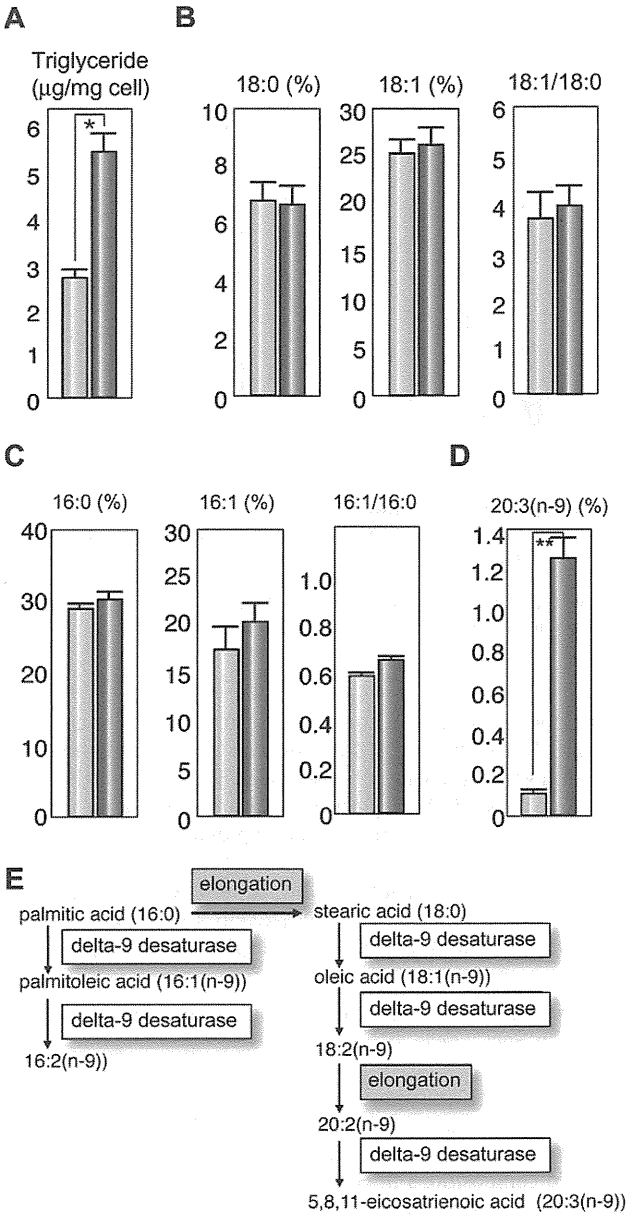

Fig. 1. Effect of the core protein on fatty acid composition in HepG2 cells. The fatty acid compositions of the total cell lipids were analyzed and the ratios of 18:1/18:0 and 16:1/16:0 in the core-expressing and control HepG2 cells were calculated. (A) Concentrations of triglycerides. (B) Percentages of stearic acid (18:0) and oleic acid (18:1(n-9)), and the 18:1/18:0 ratio. (C) Percentages of palmitic acid (16:0) and palmitoleic acid (16:1(n-9)), and the 16:1/16:0 ratio. (D) Percentage of eicosatrienoic acid (20:3(n-9). (E) Schematic display of synthetic pathway of n-9 fatty acids. Light blue bars indicate control cells and dark blue bars indicate core-expressing cells. Values represent the mean ± SE, $n = 5$ in each group. $^{*}p < 0.05$, $^{**}p < 0.01$.

Previously, we reported that the concentration of oleic acid (18:1(n-9)) was increased compared with that of stearic acid (18:0) in liver tissues of chronic hepatitis C patients as well as in those of mice transgenic for the HCV core gene [8]. Such a change may lead to increased membrane fluidity, owing to the lower melting temperature of monounsaturated fatty acids, resulting in incremental metabolism and proliferation of hepatocytes [20–22]. On the other hand, polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (20:5(n-3)) and arachidonic acid (20:4(n-6)), are known to activate the nuclear transcription of peroxisome proliferator-activated receptors (PPAR) and suppress the sterol regulatory element binding protein (SREBP)-1. While PPARγ induces delta-9 desaturase (stearoyl-CoA desaturase) gene expression, PUFAs suppresses delta-9 desaturase activity [23]. In the current study, we determined fatty acid desaturase activities by analyzing the fatty acid compositions in HepG2 cells expressing HCV core protein by chromatography. In addition, we determined whether exogenous PUFAs restore HCV-associated changes in fatty acid metabolism.

Materials and methods

Reagents

Eicosapentaenoic acid (EPA), arachidonic acid (AA), and eicosatetraynoic acid (ETYA) were purchased from Sigma Chemical (St. Louis, MO). Other chemicals were of analytical grade and purchased from Wako Chemicals (Tokyo, Japan).

Cell culture

This study was performed using 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 an empty vector, which were described previously [24], and control bulk HepG2 cells. They were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (Invitrogen), 1 mg/ml G418, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere at 37 °C in 5% CO_2. Fatty acids were dissolved in DMEM containing defatted bovine serum albumin. The ratio of fatty acids to albumin (mole/mole) was 0.7. The cells were exposed to fatty acid–albumin complexes at various concentrations for 48 h. All the experiments were repeated at least five times.

Lipid extraction, measurement of triglyceride content, and analysis of fatty acid composition

Total cell lipids were extracted by Foch's method. The cells were washed twice with phosphate-buffered saline and collected by centrifugation. The cell pellets were homogenized with 10 vole of chloroform: methanol solution (2:1), and the mixture was shaken for 5 min. The lower phase was then washed with 4 vole of saline, dried on anhydrous sodium sulfate, and evaporated to complete dryness. For the analysis of fatty acid composition, 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).

Measurement of the ketone body ratio and lactate/pyruvate

The cells were cultured to confluence on 3.5 cm dishes, and the medium was replaced with 700 μl of fresh one. After 24 h of incubation, the levels of acetoacetate and β-hydroxybutyrate in the medium were measured by monitoring the production or consumption of nicotinamide adenine dinucleotide (NADH) with Ketorex kit (Sanwa Chemical, Nagoya, Japan) [26]. The ketone body ratio (KBR) was calculated as the acetoacetate/β-hydroxybutyrate ratio. The lactate and pyruvate levels in the medium were measured at random times by the lactate oxidase method and pyruvate oxidase method, respectively.

Effect of pyruvate on lipid metabolism

In some experiments, pyruvate (Wako Chemicals) was added to culture medium at a final concentration of 0, 1, 5, or 10 mM. After 48 h of incubation at 37 °C, the cells were harvested and subjected to fatty acid composition analysis or real-time PCR analysis.

Real-time PCR

RNA was prepared from cultured cells using TRIzol LS (Invitrogen, Carlsbad, CA). The fluorescent signal was measured using ABI prism 7000 (Applied Biosystems, Tokyo, Japan). The genes encoding mouse sterol regulatory element-binding proteins (SREBP)-1a, SREBP-1c, delta-9 desaturase, and hypoxanthine phosphoribosyltransferase were amplified with the primer pairs CACAGCGGTTTTGAACGAC and CTGGCTCCTCTTTGATCCCA, ACGGAGCCATGGATTGCACATTTG and TACATCTT TAAAGCAGCGGGTGCCGATGGT, TTCCCTCCTGCAAGCTCTAC and CGCAAGAAGG TGCTAACGAAC, and CCAGCAAGCTTGCAACCTTAACCA and GTAATGATCAGTCAAC GGGGGAC, respectively.

Statistical analysis

Data are presented as the mean ± SE. The data were analyzed by Mann–Whitney *U* test. Differences were considered statistically significant when $p < 0.05$.

Results

Triglyceride content in HepG2 cells expressing HCV core protein

To validate the relationship between the lipid accumulation and the core protein, we first determined the triglyceride contents in core-protein-expressing HepG2 clones (core-expressing cells), Hep39J, Hep396, Hep397, and control HepG2 cells. Core-expressing Hep396 cells contained significantly larger amounts of triglyceride than the control cells (Fig. 1A, $p < 0.01$), which are consistent with the results of previous studies on culture cells and transgenic mice [6,7,27]. Similar results were obtained with the other core-expressing cell lines.

Fatty acid compositions of total cell lipids

Analysis on the fatty acid compositions of total lipids revealed that the concentration of oleic acid (18:1(n-9)) and the ratio of oleic acid/stearic acid (18:1/18:0) in the core-expressing cells are similar to those in the control cells (Fig. 1B). The ratio of palmitoleic acid (16:1(n-9))/palmitic acid (16:1/16:0) was higher in the core-expressing cells than that in the control cells, but the difference was not significant (Fig. 1C). This rather dissociates from the results obtained in HCV core gene transgenic mice, in which the 18:1/18:0 ratio was significantly higher than that in control mice, thereby suggesting an increased delta-9 desaturase activity as a consequence of the HCV core protein expression [8]. However, it should be noted that the concentration of 5,8,11-eicosatrienoic acid (20:3(n-9)), a downstream product of n-9 fatty acid desaturation, was approximately13 times higher in the core-expressing cells than that in the control cells (Fig. 1D and E, $p < 0.01$). This is due to the fact that the activity of the delta-6 desaturase, an enzyme downstream of delta-9 desaturase, is also high in HepG2 cells, resulting in the relatively lower concentration of 18:1 in the core-expressing cells despite the high delta-9 desaturase activity. Actually, the delta-6 desaturase activity has been shown to be inherently high in HepG2 cells [28,29].

To verify this possibility, we administered ETYA, which inhibits delta-6 desaturase activity, to the cell cultures. Because similar results were obtained with the other core-expressing HepG2 cell lines, subsequent experiments were carried out using the Hep396 cell line. The addition caused significant increases in both 18:1/18:0 and 16:1/16:0 ratios in the core-expressing cells but not in the control cells (Fig. 2A 0 vs. 10 μg/ml and 0 vs. 50 μg/ml; $p < 0.05$, respectively). When compared between the

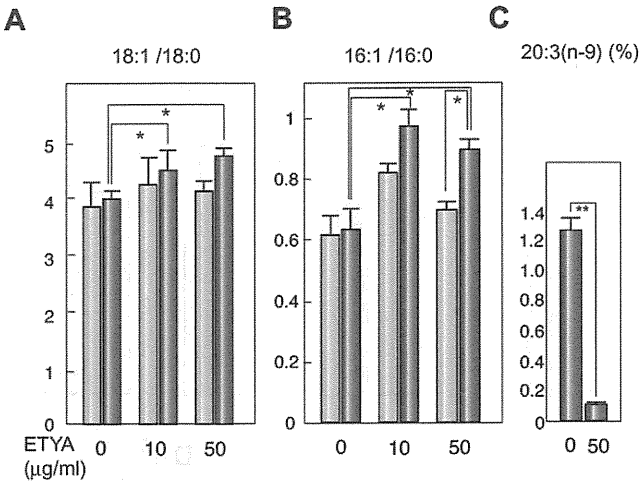

Fig. 2. Effect of ETYA on delta-9 desaturase index. HepG2 cells with or without the core protein were incubated with ETYA for 48 h. The fatty acid compositions of the total cell lipids were analyzed, and the ratios of 18:1/18:0 (A) and 16:1/16:0 (B), and the percentage of eicosatrienoic acid (20:3(n-9) (C) were computed. Light blue bars indicate control cells and dark blue bars indicate core-expressing cells. $N = 5$ in each group. *$p < 0.05$. ETYA, eicosatetraynoic acid.

core-expressing cells and control cells after the treatment with 50 μg/ml ETYA, the 18:1/18:0 ratio was higher and the 16:1/16:0 ratio was significantly higher (Fig. 2B, $p < 0.05$) in the core-expressing cells. ETYA (50 μg/ml) significantly decreased the concentration of 20:3(n-9) in the core-expressing cells (Fig. 2C, $p < 0.01$). These results suggest that the HCV core protein enhances the activities of delta-9, and possibly, delta-5 desaturases, modulating fatty acid metabolism in HepG2 cells, in which the delta-6 desaturase activity is intrinsically high (Fig. 1E) [28,29].

PUFAs modify fatty acid compositions and decrease triglyceride contents in HepG2 Cells

PUFAs are known to suppress the activities of both delta-9 and delta-6 desaturases. We, therefore, added PUFA, EPA, or AA, to the culture cell medium to examine the effect of PUFAs on the fatty acid compositions in HepG2 cells expressing the core protein. EPA and AA individually decreased the 18:1/18:0 and 16:1/16:0 ratios in a similar extent in both the core-expressing cells and the control cells (Fig. 3, $p < 0.05$). EPA and AA also significantly decreased the concentration of 20:3(n-9) in the core-expressing cells in a dose-dependent manner (Fig. 4, $p < 0.05$). In addition, EPA and AA individually decreased the triglyceride concentration in cells, in particular, in the core-expressing cells (Fig. 5, in core-expressing cells, $p < 0.01$; in control cells, $p < 0.05$, respectively).

Ketone body ratio and lactate/pyruvate ratio

Although the mechanism by which the HCV core protein enhances fatty acid desaturation is yet unclear, one possibility is the creation of an overreduced state in the core-expressing cells. The overreduced state or the accumulation of NADH in cells is known to accelerate the activities of fatty acid desaturases [30,31]. Such a condition may originate from the dysfunction of the mitochondrial electron transfer system (ETS), which has been

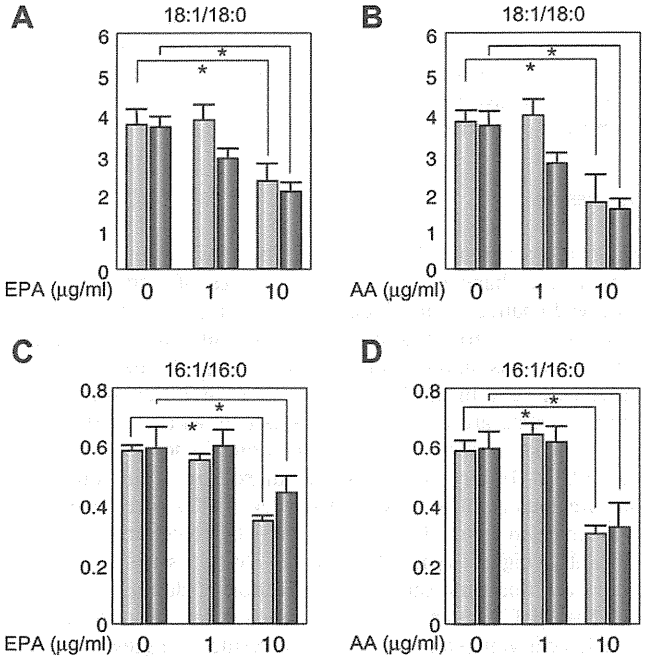

Fig. 3. Effect of EPA and AA on delta-9 desaturase index. HepG2 cells with or without the core protein were incubated with EPA (A and C) or AA (B and D) for 48 h. The fatty acid compositions of the total cell lipids were analyzed and the ratios of 18:1/18:0 (A and B) and 16:1/16:0 (C and D) were computed. Light blue bars indicate control cells and dark blue bars indicate core-expressing cells. $N = 5$ in each group. $^{*}p < 0.05$. EPA, eicosapentaenoic acid; AA, arachidonic acid.

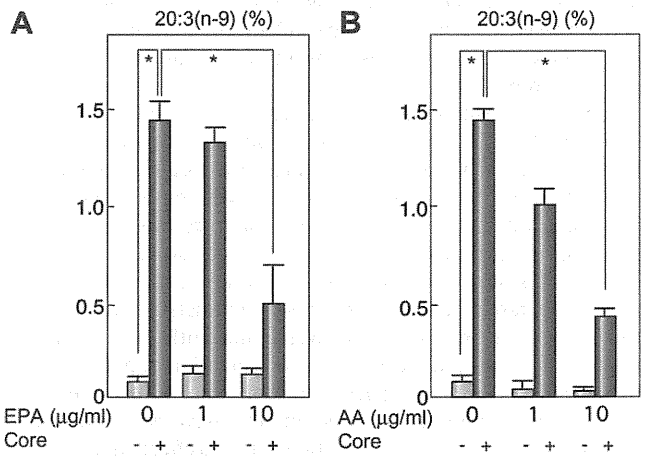

Fig. 4. Effect of EPA and AA on the concentration of 20:3(n-9). HepG2 cells with or without the core protein were incubated with EPA (A) or AA (B) for 48 h. The fatty acid compositions of the total cell lipids were analyzed and the percentages of the C20:3(n-9) fraction were measured. Light blue bars indicate control cells and dark blue bars indicate core-expressing cells. $N = 5$ in each group. $^{*}p < 0.05$.

suggested to be associated with HCV infection by the action of the HCV core protein [32–35]. Then, we explored the possibility that an increase in the NADH level, which is caused by the mitochondrial ETS dysfunction, induces the activation of fatty acid desaturases. Because fatty acid synthesis or fatty acid desaturation is accompanied by the oxidation of NAD(P)H, we measured the ketone body ratio (KBR) in the culture medium to estimate the redox state in the HepG2 cells expressing the core protein.

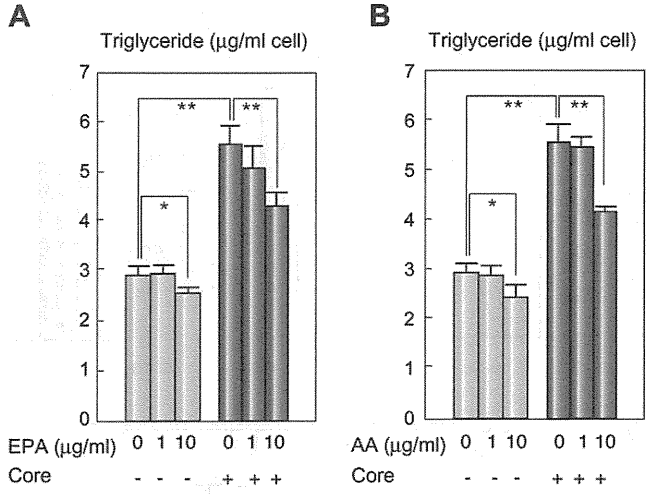

Fig. 5. Effect of EPA and AA on triglyceride content. HepG2 cells with or without the core protein were incubated with EPA (A) or AA (B) for 48 h. The triglyceride volume of the total cell lipids was measured and the triglyceride contents in the cells were calculated. Light blue bars indicate control cells and dark blue bars indicate core-expressing cells. $N = 5$ in each group. $^{*}p < 0.05$, $^{**}p < 0.01$.

The KBR, which is in equilibrium with the intramitochondrial $NAD^+/NADH$ [26,36], in the culture medium of the core-expressing cells, was significantly lower than that of control cells (Fig. 6A, $p < 0.01$). The ratio of lactate to pyruvate (lactate/pyruvate), which is proportional to the cytosolic $NADH/NAD^+$ [26], in the culture medium of the core-expressing cells was significantly higher than that of control cells (Fig. 6B, $p < 0.05$). These results, the higher $NADH/NAD^+$ ratio in both determinations, indicate that NADH accumulates in the core-expressing HepG2 cells, resulting in the overreduced state, as a consequence of the core protein expression. The amounts of total ketone bodies were significantly higher in the core-expressing cells than that in the control cells (Fig. 6C).

Effects of pyruvate on lipid metabolism in core-expressing cells

The addition of pyruvate into this constitutive core protein expression system, in which the pyruvate metabolism is in equilibrium, is expected to cause a reduction in the NADH level along with increases in the levels of lactate and NAD^+, because pyruvate tends to be converted to lactate by the action of lactate dehydrogenase (LDH) under the condition of high $NADH/NAD^+$ ratio [26,36]. Actually, the addition of pyruvate into the culture medium at various concentrations increased the KBR and reduced the amount of 5,8,11-eicosatrienoic acid (20:3 (n-9)) (Fig. 6D, $p < 0.05$ at 10 mM pyruvate), while it had no effect on the control cells. It also caused a reduction in the amount of triglyceride in the core-expressing cells but not in the control cells (Fig. 6E). This finding strongly supports the notion that NADH accumulation is, at least, one of the causes of the activation of fatty acid desaturases in this HCV model. The mRNA levels of anti-oxidant genes significantly decreased after the incubation with pyruvate at 10 mM (catalase, 1.27 ± 0.06 vs. 0.91 ± 0.05; glutathione synthetase 1.39 ± 0.04 vs. 1.01 ± 0.06; glutathione peroxidase 1.48 ± 0.03 vs. 1.23 ± 0.07, pyruvate (−) vs. pyruvate (+), $p < 0.05$, respectively), suggesting that pyruvate reduced the levels of oxidative stress in the core-expressing HepG2 cells.

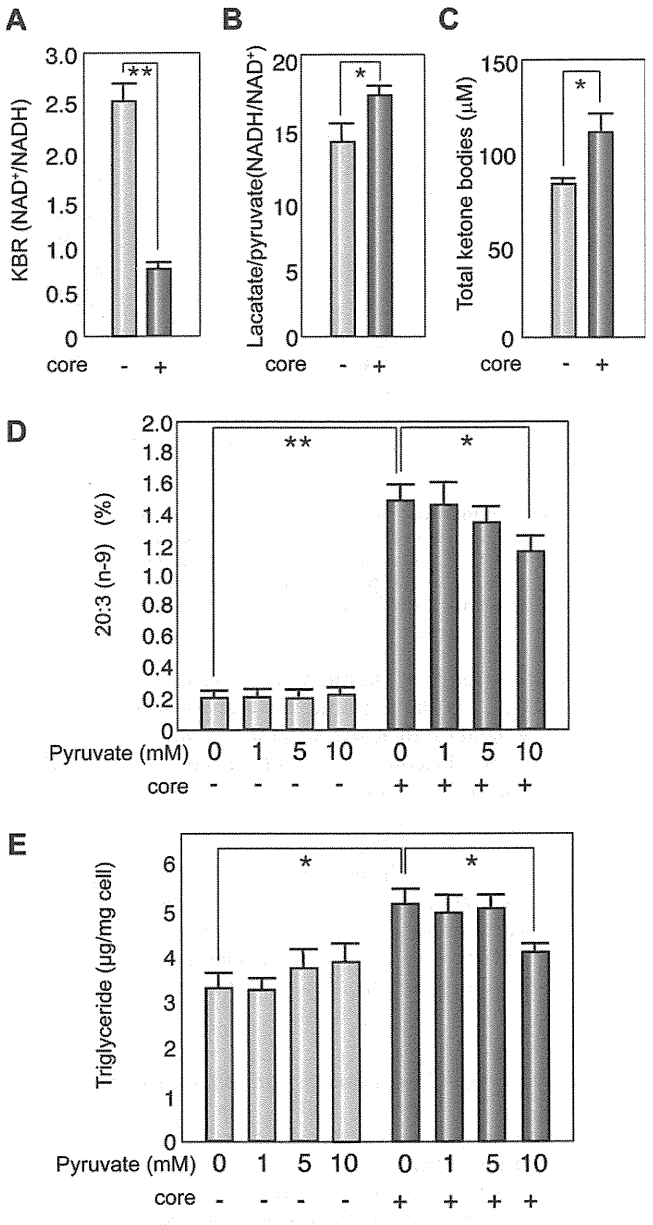

Fig. 6. NADH accumulation and effect of pyruvate in core-expressing cells. HepG2 cells with or without the core protein were subjected to the determination of ketone body ratio (A) and lactate/pyruvate ratio (B) for the precise estimation of NAD^+/NADH and NADH/NAD^+. (C) Total ketone bodies. (D) The percentages of the C20:3(n-9) fraction were measured after incubation with pyruvate at various concentrations. (E) The total amount of triglyceride was measured after incubation with pyruvate at various concentrations. Light blue bars indicate control cells and dark blue bars indicate core-expressing cells. $N = 5$ in each group. $^*p < 0.05$, $^{**}p < 0.01$.

Expression of SREBP-1 and desaturase genes in core-expressing cells

We previously showed that the core protein activates the expression of the SREBP-1c gene, which regulates the production of triglyceride [37] in the liver. We, therefore, examined the mRNA levels of genes associated with lipid metabolism in the current system. As shown in Fig. 7, the mRNA levels of SREBP-1c and delta-9 (stearoyl CoA) desaturase genes, but not that of the SREBP-1a gene, were significantly higher in the core-expressing cells than that in the control cells. Of note, the mRNA levels of the former two genes significantly decreased after the incubation with AA. The treatment with pyruvate also reduced the mRNA levels of the two genes, but the difference was not statistically significant compared with the control.

Discussion

The core protein of HCV modulated the activities of delta desaturases and changed the saturation states of fatty acids. The observed change in the HepG2 cells, namely, an increase in the amounts of unsaturated fatty acids, may support cell proliferation, by increasing the fluidity of the cell membrane as reported previously [20]. In the HepG2 cells expressing the core protein, the delta-6 desaturase activity was as high as that of the delta-9 desaturase, leading to the accumulation of a downstream product, 20:3(n-9) fatty acid. This was, unexpectedly, in contrast to our previous result on the liver tissues of HCV core gene transgenic mice, in which the 18:1/18:0 and 16:1/16:0 ratios were significantly higher than that in the liver tissues of normal littermate mice, indicating the activation of delta-9 desaturase [8]. The 16:1/16:0 and 18:1/18:0 ratios observed in the control HepG2 cells were consistent with the results of a previous study: the delta-6 desaturase activity is inherently higher in HepG2 cells than in normal mouse hepatocytes [28,29]. This may explain the difference in the effect of the core protein on lipid metabolism in these two systems, namely, HepG2 cells and mouse liver tissues. The significant increase in the delta-9 desaturase index and high concentration of 20:3(n-9) by the administration of ETYA, a delta-6 desaturase inhibitor, indicate the activation of delta-9 desaturase in the core-expressing cells. The results of real-time PCR analysis for determining the mRNA levels of these enzymes corroborated the current estimation of desaturase activities as determined by fatty acid analysis.

The mechanism underlying the activation of fatty acid desaturation by the HCV core protein is still unclear, but one possibility is the presence of an overreduced state in the core-expressing cells. The HCV core protein is closely associated with mitochondrial dysfunction, in particular, that of the respiratory chain complexes, resulting in an impairment of NADH oxidation [32–35]. NADH accumulation leads to an increase in desaturase activities through the augmentation of microsomal electron transfer [38]. In fact, the KBR in the core-expressing cells was significantly lower than that in the control cells, indicating the accumulation of NADH within the cells. The addition of pyruvate resulted in an increase in the KBR and a reduction in the amounts of triglyceride and 5,8,11-eicosatrienoic acid (20:3 (n-9)) while it had no effect on the control cells, strongly supporting the notion that NADH accumulation induced by the core protein is, at least, one of the causes of the activation of fatty acid desaturases in this HCV model.

Another possible mechanism underlying the accelerated desaturation is the activation of SREBP-1c, which controls the expression of delta-9 desaturase. In fact, the level of SREBP-1c mRNA was higher in the core-expressing cells than that in the control cells as reported previously [37]. The relief of NADH accumulation by pyruvate administration resulted in the reduced accumulation of triglyceride and unsaturated fatty acids, which was accompanied by the reduction in SREBP-1c and delta-9 desaturase gene expression levels. The intracellular accumulation of NADH might be involved in the activation of the SREBP-1c gene expression by the core protein. Thus, NADH accumulation, which

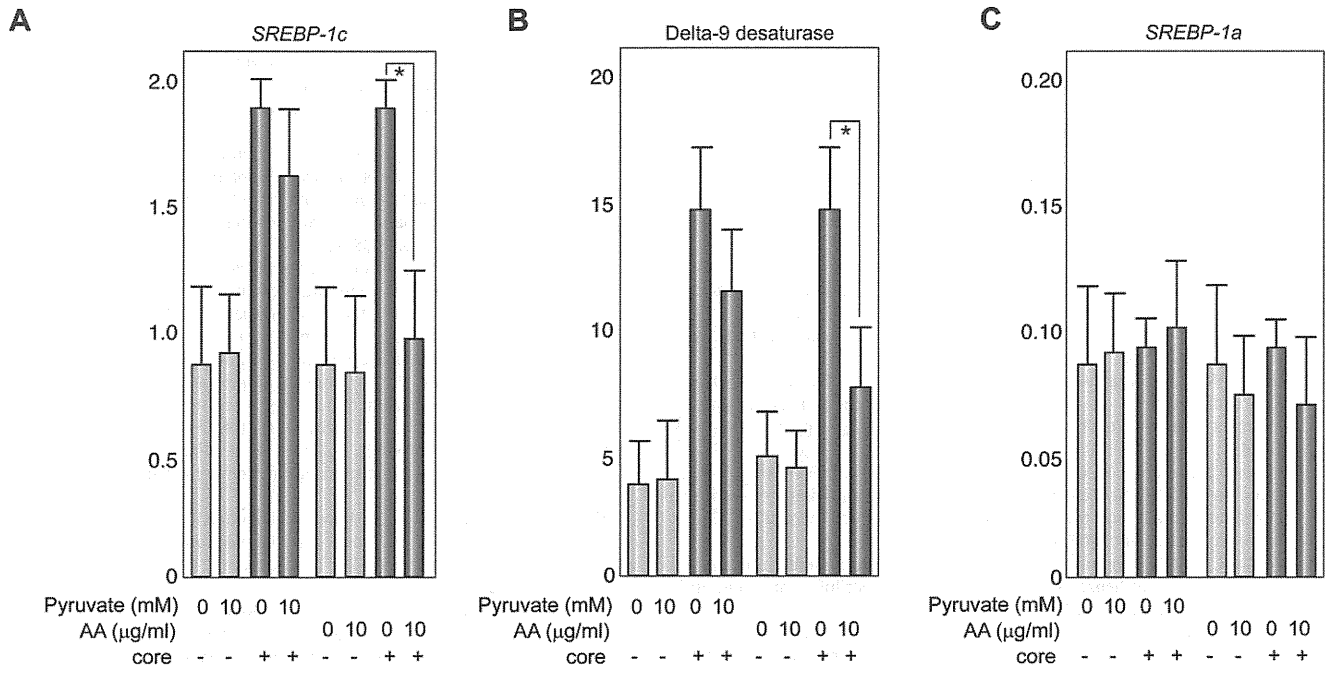

Fig. 7. Effect of pyruvate and AA on mRNA levels of lipid-associated genes. The mRNA levels of *SREBP-1c* (A), delta-9 desaturase (B) and *SREBP-1a* (C) genes were determined by real-time PCR analysis. The transcription of the genes was normalized with that of hypoxanthine phosphoribosyltransferase, and the values are expressed as relative activities. Light blue bars indicate control cells and dark blue bars indicate core-expressing cells. $N = 5$ in each group. $^*p < 0.05$. SREBP, sterol regulatory element binding protein.

is induced by the core protein through the impairment of the mitochondrial complex function [35], may be a key event that leads to the SREBP-1c activation, the desaturase activation, and the development of steatosis associated with HCV infection.

EPA and AA (PUFAs), which are known to suppress desaturase activities, lowered the 18:1/18:0 and 16:1/16:0 ratios and decreased the concentration of 20:3(n-9) concomitantly with that of triglyceride, regardless of the presence of the core protein, probably through SREBP-1c suppression (Fig. 7) [39]. On the other hand, the administration of EPA or AA did not affect the KBR in the core-expressing or control cells (data not shown), limiting the PUFAs ability to counteract the effect of the core protein. This is in contrast to the fact that the addition of pyruvate caused an increase in the KBR and a reduction in the amounts of triglyceride and 5,8,11-eicosatrienoic acid (20:3 (n-9)), while it had no effect on the control cells.

Fatty acid desaturation is closely associated with increased membrane fluidity [20], leading to augmented cell metabolism and higher cell division rates [21,22]. Although the relationship between carcinogenesis and lipid metabolism altered by the HCV core protein remains to be further clarified, alterations in lipid metabolism, in particular, in the desaturation of fatty acids, are closely associated with HCV infection, and PUFAs could prevent the pathogenesis of HCV-associated disorders involving lipid metabolism.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Hepatitis C Virus Core Protein Compromises Iron-Induced Activation of Antioxidants in Mice and HepG2 Cells

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One of the characteristics of hepatitis C virus (HCV) infection is the unusual augmentation of oxidative stress, which is exacerbated by iron accumulation in the liver, as observed frequently in hepatitis C patients. Using a transgenic mouse model, the core protein of HCV was shown previously to induce the overproduction of reactive oxygen species (ROS) in the liver. In the present study, the impact of iron overloading on the oxidant/antioxidant system was examined using this mouse model and cultured cells. Iron overloading caused the induction of ROS as well as antioxidants. However, the augmentation of some antioxidants, including heme oxygenase-1 and NADH dehydrogenase, quinone 1, was compromised by the presence of the core protein. The attenuation of iron-induced augmentation of heme oxygenase-1 was also confirmed in HepG2 cells expressing the core protein. This attenuation was not dependent on the Nrf2 transcription factor. Thus, HCV infection not only induces oxidative stress but also hampers the iron-induced antioxidant activation in the liver, thereby exacerbating oxidative stress that would facilitate hepatocarcinogenesis. ***J. Med. Virol. 82:776–782, 2010.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: oxidative stress; transgenic mouse; hepatocellular carcinoma; antioxidant; heme oxygenase-1

INTRODUCTION

Hepatitis C virus (HCV) is a major cause of liver disease. Persistent HCV infection leads to the development of chronic hepatitis, cirrhosis, and, eventually, hepatocellular carcinoma (HCC), thereby being a serious problem both in medical and socio-economical aspects [Saito et al., 1990]. Despite overwhelming evidence from epidemiological studies, the precise mechanism of hepatocarcinogenesis in HCV infection is still not fully understood. Recently, it has been shown that the core protein of HCV induces HCC in transgenic mice [Moriya et al., 1998; Naas et al., 2005; Machida et al., 2006]. Augmentation of oxidative stress is implicated in the pathogenesis of liver disease in HCV infection as shown by a number of clinical and basic studies [Farinati et al., 1995; Moriya et al., 2001; Choi and Ou, 2006]. Reactive oxygen species (ROS) are endogenous oxygen-containing molecules formed as normal products during aerobic metabolism. ROS can induce genetic mutations as well as chromosomal alterations and thus contribute to cancer development in multistep carcinogenesis [Fujita et al., 2008]. Recent studies have shown that oxidative stress is more augmented in hepatitis C than in other types of hepatitis such as hepatitis B [Farinati et al., 1995; Chapoutot et al., 2000]. On the other hand, in chronic hepatitis C, HCC and fibrosis are closely associated with the amount of iron in the liver. Iron depletion both in the form of dietary iron restriction or phlebotomy improved hepatic inflammation and lowered serum aminotransferase levels in hepatitis C patients. Phlebotomy decreases the hepatic content of 8-OH deoxyguanosine, a marker of DNA damage, improved inflammation and fibrosis

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; ROS, reactive oxygen species; HO-1, heme oxygenase-1; thiobarbituric acid reactive substances (TBARS); GST, glutathione-*S*-transferase; SOD, superoxide dismutase; GPx, glutathione peroxidase; NQO1, NAD(P)H dehydrogenase, quinone 1; AP-1, activator protein-1; NF-κB, nuclear factor-kappa B; Bach1, BTB and CNC homology 1.

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scores, and prevented HCC development [Kato et al., 2001].

Thus, a major role in the pathogenesis of HCV-associated liver disease has been attributed to oxidative stress augmentation, in association with iron accumulation, but no underlying mechanism is understood well yet. Hence, it is an important issue to understand the mechanism of oxidative stress augmentation, which may allow us to develop new tools of therapies for chronic hepatitis C. Iron accumulation and oxidant/antioxidant status were assessed with or without iron overloading in the liver of a transgenic mouse model of HCC in HCV infection. The expression levels of genes associated with the antioxidant system were also determined.

MATERIALS AND METHODS

Transgenic Mice and Cultured Cells

The production of HCV core gene transgenic mice has been described previously [Moriya et al., 1998]. Briefly, the core gene of genotype 1b HCV was introduced into C57BL/6 mouse embryos (Clea Japan, Tokyo, Japan). Mice were cared for according to institutional guidelines approved 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. At least five mice were used for each experiment and the data were subjected to statistical analysis.

Determination of Iron in the Liver

Determination of Fe in the liver was performed by Shimadzu Techno-Research, K.K. (Kyoto, Japan) using Inductively Coupled Plasma apparatus, ICP8100 (Shimadzu Corp.). Briefly, samples were resolved by microwave (Microwave Resolution System ETHOS-TC, Milestone General, Inc., Tokyo, Japan) after the addition of nitric acid, and the volume was adjusted by the addition of H_2O. This solution was then subjected to the quantification of iron using ICP8100 (http://www.shimadzu.com/products/lab/ms/glossary/oh80jt00000008w4.html).

Iron Loading Experiments

For the short-term iron loading experiment, three doses of $FeSO_4$ solution (100 mg/kg BW, suspended in dH_2O) or vehicle only were administered to the core gene transgenic or control mice i.p., with intervals of 24 hr at the age of 6 months [Zhu and Miller, 2007]. For the long-term iron loading experiment, 50 mg/kg BW of $FeSO_4$ was administered to the core gene transgenic mice i.p., once a week for 3 months from the age of 3 months. 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 [Ruggieri et al., 2004]. For the iron loading experiments, hemin solution (10 mM in DMSO) was added to the culture medium at the final concentration of 5 μM, and the cells were incubated for 5 or 72 hr.

Evaluation of Oxidant and Antioxidant Systems

Lipid peroxidation in the liver was estimated spectrophotometrically using thiobarbituric acid reactive substances (TBARS) and is expressed in terms of malondialdehyde formed per milligram protein. In the cell culture experiment, the cells were examined for ROS production using chloromethyl 2′,7′-dichlorodihydrofluorescein diacetate (Molecular Probes, Inc., Eugene, OR). For the evaluation of DNA damage in cells, apurinic/apyrimidinic (AP) sites were determined using a DNA Damage Quantification Kit (Dojindo Molecular Technologies, Inc., Tokyo, Japan).

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 fluorescence signal was measured using ABI Prism 7000 (Applied Biosystems, Tokyo, Japan). Primers and probes for hepcidin (Unigene ID: Mm. 439939), catalase (Mm. 4215), glutathione-*S*-transferase (GST) (Mm. 1090), superoxide dismutase (SOD)1 (Mm. 01344233), glutathione peroxidase (GPx)1 (Mm. 1090), heme oxygenase (HO)-1 (Mm. 00516004), NAD (P)H dehydrogenase, quinone (NQO) 1 (Mm. 500821), activator protein (AP)-1 (Mm. 275071), nuclear factor-kappa B (NF-κB)1 (Mm. 256765), BTB and CNC homology (Bach)1 (Mm. 26147), and hypoxanthine phosphoribosyltransferase (Mm. 299381) were purchased as assays-on-demand (Applied Biosystems). Each cDNA prepared was used in triplicate for the real-time PCR procedures for each gene tested.

Western blotting was performed with an anti-HO-1 antibody (Stressgen Biotechnologies, Corp., Victoria, BC, Canada) or anti-Nrf2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and Super Signal Femto (Pierce, Rockford, IL).

Statistical Analysis

Data are presented as the mean ± SE. The significance of the difference in means was determined by Mann–Whitney's *U*-test. $P < 0.05$ was considered significant.

RESULTS

Iron Accumulation in Ordinarily Fed Core Gene Transgenic Mice

The core gene transgenic mice develop HCC after an incubation period of approximately 16 months, in the absence of inflammation [Moriya et al., 1998]. During the incubation period, there is augmentation of oxidative stress with a concomitant activation of antioxidants and development of DNA damage in the liver [Farinati

et al., 1995]. For mice fed with normal chow, the concentration of total iron in the liver was higher in the core gene transgenic mice than in the control mice, and the difference became significant after the age of 12 months (Fig. 1A). The level of hepcidin mRNA, the product of which maintains iron homeostasis by a direct inhibition of ferroportin [Muckenthaler, 2008], was significantly higher in the core gene transgenic mice than in the control mice at the age of 3 and 15 months (Fig. 1B).

Iron Overloading to Core Gene Transgenic Mice

When the mice were overloaded with iron, the intrahepatic levels of iron markedly increased. In the short-term iron loading (for three consecutive days at the age of 6 months), the iron concentration in the iron-treated mice was more than twofold higher than that in the vehicle-treated ones in both the core gene transgenic and control mice, but there was no difference between the core gene transgenic and control mice. In the long-term iron loading (for 3 months from the age of 3 months), the iron concentration became significantly higher in the core gene transgenic mice than in the control mice (Fig. 1C). The hepcidin mRNA level was proportionally higher in the long-term iron-loaded mice than in the vehicle-treated mice and was significantly higher in the core gene transgenic than in the control mice ($P < 0.05$, Fig. 1D), suggesting that the positive feedback from iron to hepcidin is instrumental.

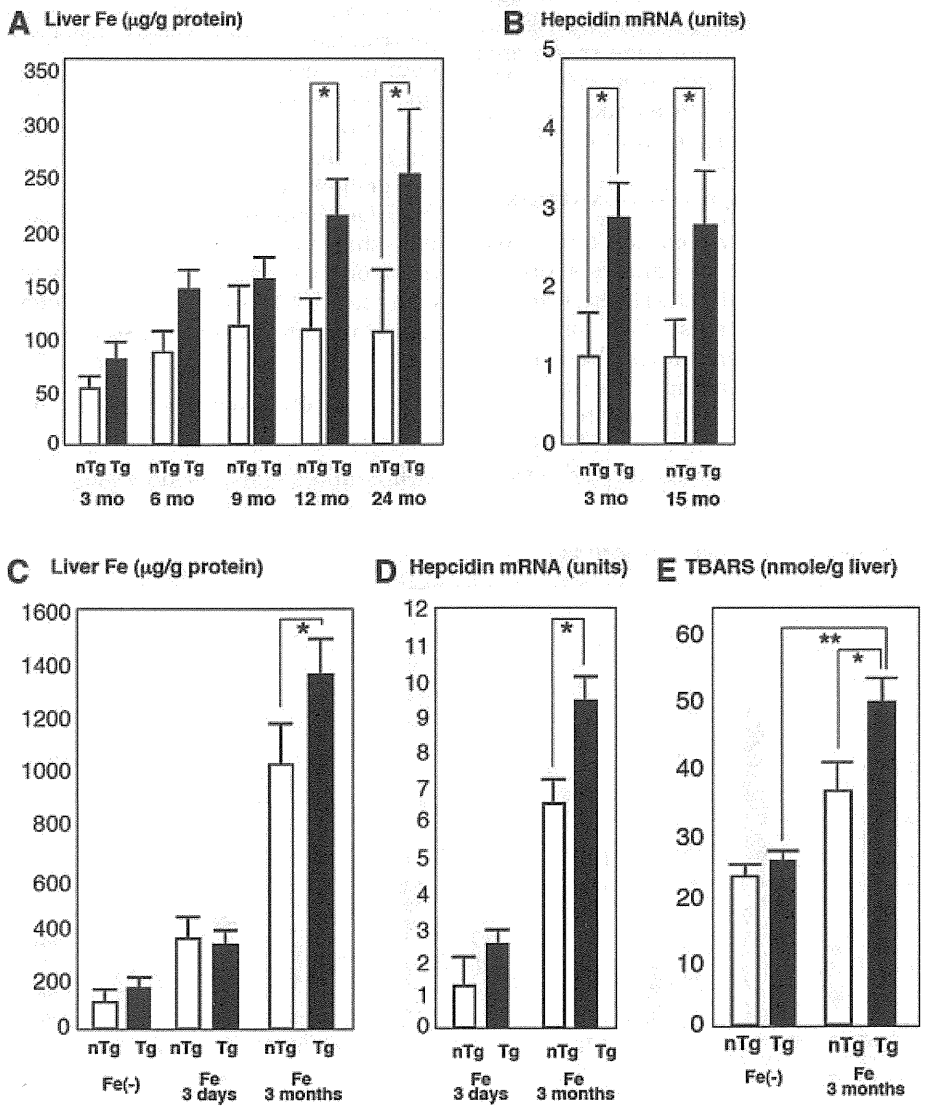

Fig. 1. **A**: Amounts of iron accumulated in the liver of mice fed with normal chow. **B**: Hepcidin mRNA level in the liver of mice fed with normal chow at 3 and 15 months. **C**: Amounts of iron in the liver of mice subjected to iron overloading for 0, 3 days, or 3 months. **D**: Hepcidin mRNA level in the liver of mice subjected to iron overloading for 0, 3 days, or 3 months. **E**: Oxidative stress in the liver of mice subjected to iron overloading for 0 or 3 months. The data represent means ± SE, n = 5 in each group. $^{*}P < 0.05$, $^{**}P < 0.01$. nTg, nontransgenic control mice; Tg, transgenic mice; TBARS, thiobarbituric acid reactive substances.

Oxidative Stress and Iron Overloading in Mice

As described previously, ROS production in the liver of the core gene transgenic mice is already augmented at the young age, but the lipid peroxidation level is not higher than that in the control mice due to the concomitant activation of antioxidant system [Moriya et al., 2001]. This was also the case in the current experiment: there was no significant difference in the ROS level at the age of 6 months between the core gene transgenic and control mice that were treated with the vehicle, as determined by TBARS (Fig. 1E). However, after the long-term iron treatment, ROS levels in the liver of the core gene transgenic mice became significantly higher than that in the control mice (Fig. 1E, $P < 0.05$). After the long-term iron treatment, the AP site index, a marker for nuclear DNA damage, became significantly higher in the core gene transgenic mice than in the vehicle-treated core gene transgenic mice (5.2 ± 0.6 vs. 3.9 ± 0.3 nmol/g liver, $P < 0.05$), showing that iron overloading facilitates nuclear DNA damage through oxidative stress augmentation.

Impact of Iron Overloading on Antioxidants

The effect of long-term iron overloading on antioxidants was evaluated by real-time PCR analysis (Fig. 2). As already reported [Moriya et al., 2001], the levels of antioxidant enzymes, such as catalase, GST, or SOD1, were higher in the core transgenic mice than in the control mice before iron overloading. The levels of antioxidant mRNAs were higher in the long-term iron-treated mice than in the vehicle-treated mice. However, the magnitude of augmentation by iron overloading was different among the antioxidant genes. While catalase and GST genes were significantly more enhanced in the core gene transgenic mice than in the control mice by iron overloading ($P < 0.05$), there was less augmentation in the expressions of HO-1 and NQO-1 genes in the core gene transgenic mice than in the control mice. The level of HO-1 mRNA in the long-term-treated mice was significantly higher in the control mice than in the core gene transgenic mice ($P < 0.05$, respectively), in contrast to that of catalase, GST, or SOD1 gene. The level of NQO-1 mRNA in the liver was lower in the core gene transgenic than in the control mice, although the difference was not statistically significant.

To confirm this observation, the protein level of HO-1 was determined by Western blotting. As shown in Figure 3A, there was only a marginal expression of HO-1 protein in the untreated core gene transgenic and nontransgenic mice. After the short-term iron overloading, there was a marked induction of HO-1 protein but there was no significant difference between these two. However, after the long-term overloading, there was an attenuation in the levels of HO-1 protein in both the core gene transgenic and control mice; in particular, the HO-1 level was lower in the core gene transgenic mice than in the control mice. Thus, the long-term in vivo iron overloading compromised some antioxidants, such as HO-1 and NQO-1, which may lead to an augmentation of oxidative stress in HCV infection.

HO-1 and Iron in Cultured Cells

HepG2 cells expressing the core protein were treated with hemin as described in the Materials and Methods

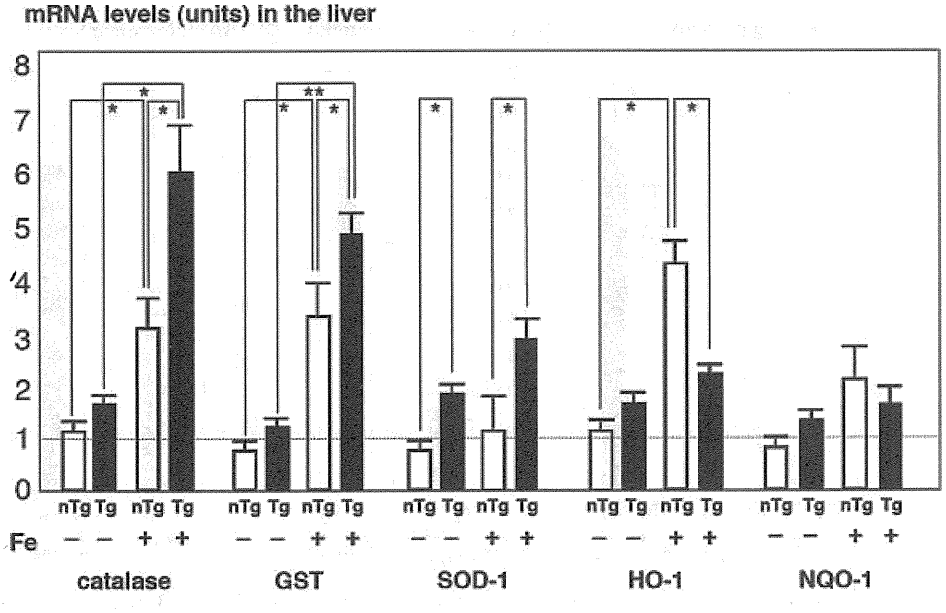

Fig. 2. Levels of antioxidant mRNA in the liver of mice subjected to iron overloading. Levels of mRNA were determined in nontransgenic control mice (open bars) or in transgenic mice (closed bars) with 3-month administration of iron (Fe+) or without it (Fe−). Mice were sacrificed at 6 months and liver tissues were subjected to determination. The data represent means ± SE, n = 5 in each group. *$P < 0.05$, **$P < 0.01$. nTg, nontransgenic control mice; Tg, transgenic mice; GST, glutathione-*S*-transferase: SOD1, superoxide dismutase 1; HO-1, heme oxygenase 1; NQO-1, NAD(P)H dehydrogenase, quinone 1.

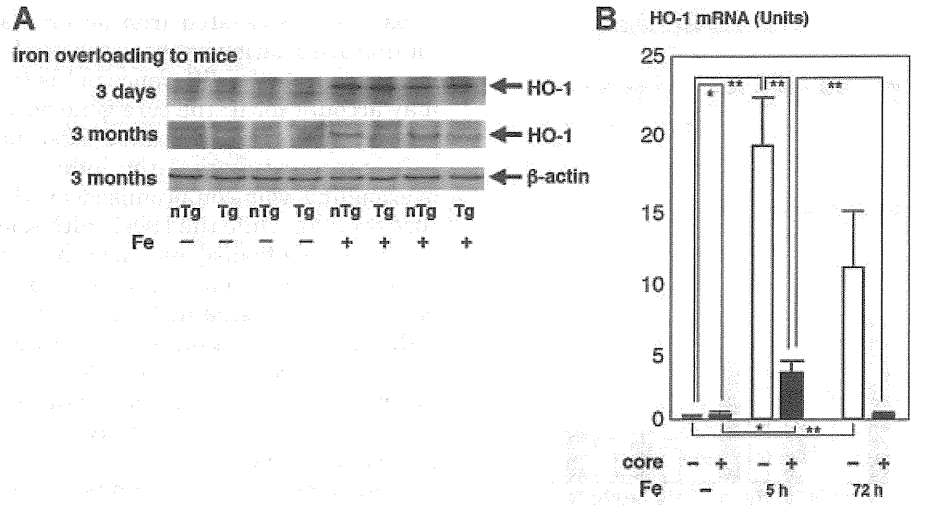

Fig. 3. **A**: Levels of HO-1 protein determined by Western blotting in the liver of mice subjected to iron overloading for 0, 3 days, or 3 months. **B**: Levels of HO-1 mRNA in core-protein-expressing or nonexpressing HepG2 cells subjected to iron loading for 0, 5, or 72 hr. nTg, non-transgenic control mice; Tg, transgenic mice; HO, heme oxygenase.

Section. Before the treatment, the HO-1 mRNA level was significantly higher in the core-expressing cells than in the control HepG2 cells (Fig. 3B, 1.00 ± 0.33 vs. 0.19 ± 0.10 arbitrary units, $P < 0.05$). After the short-term iron treatment (for 5 hr), the HO-1 mRNA levels increased in both cell lines but it was significantly higher in the control cells than in the core-expressing cells (Fig. 3B, $P < 0.01$). After the long-term iron treatment (72 hr), there was a decrease in the HO-1 mRNA levels in both cell lines compared with those after the short-term iron treatment, but the magnitude of decrease was marked in the core-expressing cells ($P < 0.05$, 5 hr core (+) vs. 72 hr core (+)). Thus, the core protein compromised the iron-induced enhancement of HO-1, and such an effect of the core is more prominent in the long-term iron overloading than in the short-term one. Thus, both in mice and cultured cells, the iron-induced induction of HO-1 was compromised by the presence of the core protein. Similar to the iron-overloading experiment in mice, iron treatment induced ROS production in cultured cells to a greater extent in the core-expressing cells than in control cells (data not shown).

Absence of Nrf2 Involvement in HO-1 Impairment by the Core Protein

To explore the mechanism underlying the differential responses to iron overloading in antioxidant gene expressions, the intracellular distribution of a transcription factor, Nrf2, which regulates the expression of HO-1 [Srisook et al., 2005; Farombi and Surh, 2006] was examined. For this analysis, the liver tissues from the core gene transgenic and control mice, either iron-overloaded or not, were subjected to subcellular fractionation, followed by detection by Western blotting. However, there was no decrease or rather an increase in the Nfr2 level localized in the nuclear fraction after the iron-overloading treatment in the core transgenic mouse liver (Fig. 4A), indicating that the attenuation of HO-1 expression is not dependent on Nrf2. Finally, no interaction was observed between the core protein and the Nrf2 protein as determined by coimmunoprecipitation using cultured cells (data not shown). Because transcription factors, other than Nrf2, such as AP-1 and NF-κB, may be responsible for the HO-1 gene expression [Ferrándiz and Devesa, 2008], and Bach1, an HO-1 repressor [Shan et al., 2004], may be responsible for the attenuation of HO-1 expression, changes in expression levels of these factors were determined by the real-time PCR. AP-1 was activated by the core protein while NF-κB was not in the liver of transgenic mice [Tsutsumi et al., 2002]. With the administration of iron, mRNA levels of AP-1 and NF-κB1 were increased slightly both in core gene transgenic and control mice (Fig. 4B), thereby not explaining the attenuation of iron-induced HO-1 induction by the core protein. Bach1 expression level was not changed significantly by iron administration in the core gene transgenic mice (Fig. 4B), negating the possibility that this repressor acts to inhibit the induction of HO-1 gene expression by iron.

DISCUSSION

Chronic hepatitis C is characterized by its prominent augmentation of oxidative stress [Choi and Ou, 2006; Fujita et al., 2008; Moriya et al., 2001]. Iron accumulation in the liver has been shown to aggravate the oxidative stress as shown by the increase in the amount of DNA adducts in the liver [Chapoutot et al., 2000; Moriya et al., 2001; Choi and Ou, 2006; Fujita et al., 2008]. In this study, iron was accumulated in the liver of the HCV core gene transgenic mice, which is destined to develop HCC after a certain period with increased

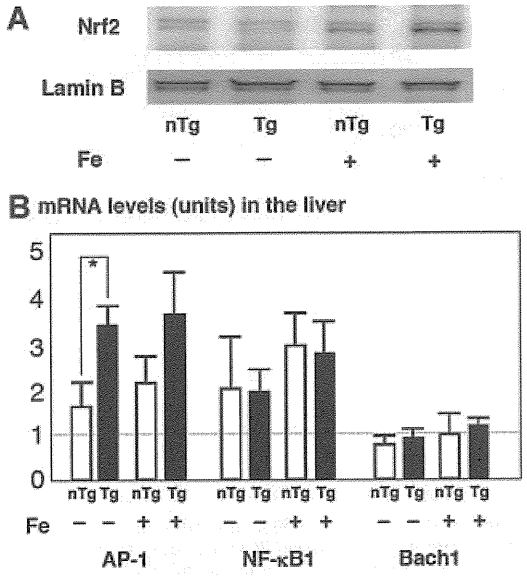

Fig. 4. **A**: Intracellular localization of Nfr2 in the liver of mice subjected to iron overloading for 0 or 3 months. The nuclear fraction was isolated from liver tissues and subjected to Western blotting with antibody to Nrf2 or lamin B (control). Levels of antioxidant mRNA in the liver of mice subjected to iron overloading. **B**: Levels of transcription factor mRNA were determined in nontransgenic control mice (open bars) or in transgenic mice (closed bars) with 3-month administration of iron (Fe+) or without it (Fe−). Mice were sacrificed at 6 months and liver tissues were subjected to determination. The data represent means ± SE, n = 5 in each group. $^*P < 0.05$. nTg, nontransgenic control mice; Tg, transgenic mice; AP-1, activator protein-1; NF-κB1, nuclear factor-kappa B1; Bach1, BTB and CNC homology 1.

oxidative stress and steatosis [Moriya et al., 1998, 2001]. Some of the key antioxidant enzymes, HO-1 and NQO-1, were not augmented sufficiently by iron overloading, while other antioxidant enzymes such as catalase and GST were augmented more strongly in the iron-overloaded core gene transgenic mice than in the iron-overloaded control or noniron-overloaded core gene transgenic mice.

The accumulation of iron observed in the liver of the core gene transgenic mice fed with normal chow corroborates well the observation in chronic hepatitis C patients [Farinati et al., 1995; Chapoutot et al., 2000; Kato et al., 2001]. Recently, it has been reported that the expression level of hepcidin, which regulates iron metabolism by inhibiting iron absorption from the intestine and utilization from the reticuloendothelial system [Muckenthaler, 2008], is decreased in the liver of mice that are transgenic for the entire HCV genome [Nishina et al., 2008] or hepatitis C patients [Fujita et al., 2007], and is implicated in iron accumulation. ROS overproduction in the liver of the entire HCV genome transgenic mice is suggested to be responsible for the decrease in the hepcidin level [Nishina et al., 2008]. In the current study, however, the level of hepcidin mRNA was significantly higher in the liver of the core gene transgenic mice than that of the control mice despite the augmentation of oxidative stress. In the current model, hepcidin seems to be instructive in the regulation of iron homeostasis and cannot be a cause of hepatitis-C-associated iron accumulation. The reason for this dissociation between the two HCV mouse models is unclear, but the difference in the transgene construct may account for it: the core gene only or the full coding sequence [Moriya et al., 1998; Nishina et al., 2008].

It is interesting that the induction of HO-1 by iron overloading was compromised by the presence of the core protein, while the HO-1 mRNA level was higher in the core gene transgenic mice than in the control mice before iron treatment. This was also observed in the case of another antioxidant, NQO-1 [Nioi and Hayes, 2004], although its induction by iron in the control mice was not as marked as that of HO-1. HO-1 is an inducible cytoprotective enzyme that catalyzes the initial and rate-limiting reaction in heme catabolism and cleaves pro-oxidant heme to form biliverdin with the release of carbon monoxide. Biliverdin is converted to bilirubin in mammals, both of which have been known to have very strong antioxidant activities [Stocker et al., 1987]. Thus, HO-1 is an antioxidant defense enzyme that converts potentially toxic heme into antioxidants. In addition, HO-1 has been also suggested to be a central antioxidant under the condition of glutathione depletion [Oguro et al., 1998]. Therefore, HO-1 is an essential protective endogenous mechanism against oxidative stress, particularly, in the case of iron overload, although it is hard to compare the abilities of antioxidants quantitatively [Srisook et al., 2005; Farombi and Surh, 2006]. Therefore, it is probable that the attenuation of HO-1 and NQO-1 would hamper the antioxidant system and lead to a robust production of oxidative stress in HCV infection. There have been some contradicting reports on the interaction between HCV and HO-1. Abdalla et al. [2004] reported that the HCV core protein down-regulated HO-1 expression, while Ghaziani et al. [2006] insist that HCV proteins including the core up-regulates HO-1 expression. HO-1 has recently been shown to suppress the propagation of HCV replicons [Zhu et al., 2008] and might be an essential molecule in the pathogenesis of HCV infection.

The mechanism underlying the compromise of iron-induced HO-1 gene augmentation by the core protein is unclear. HO-1 gene has a regulatory element that is controlled by a transcription factor Nrf2 stress [Srisook et al., 2005; Farombi and Surh, 2006]. Therefore, if the subcellular distribution of Nrf2 was altered, that is, nuclear transport were disturbed, it would explain this compromised phenomenon. However, this was not the case in the present experiment. The fact that the GST gene, the expression of which was augmented substantially by the iron overloading, also has a binding site for Nrf2 also negates the involvement of Nfr2 in the compromise by the core protein of iron-induced HO-1 gene augmentation. It has been suggested that complex intracellular signaling cascades mediate the expression of HO-1 in response to external stimuli. Transcription factors, other than Nrf2, such as AP-1 and NF-κB [Ferrándiz and Devesa, 2008] and Bach1, an HO-1 repressor [Shan et al., 2004], may be responsible for the HO-1 gene expression, but these factors did not explain

the current attenuation of HO-1 by the core protein under iron stimulation. Identification of the mechanism may lead to the development of new therapeutic devices with the relief of the core-induced compromise of the iron-induced augmentation of HO-1, which may strengthen the antioxidant system and suppress HCV replication.

ACKNOWLEDGMENTS

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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

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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-

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