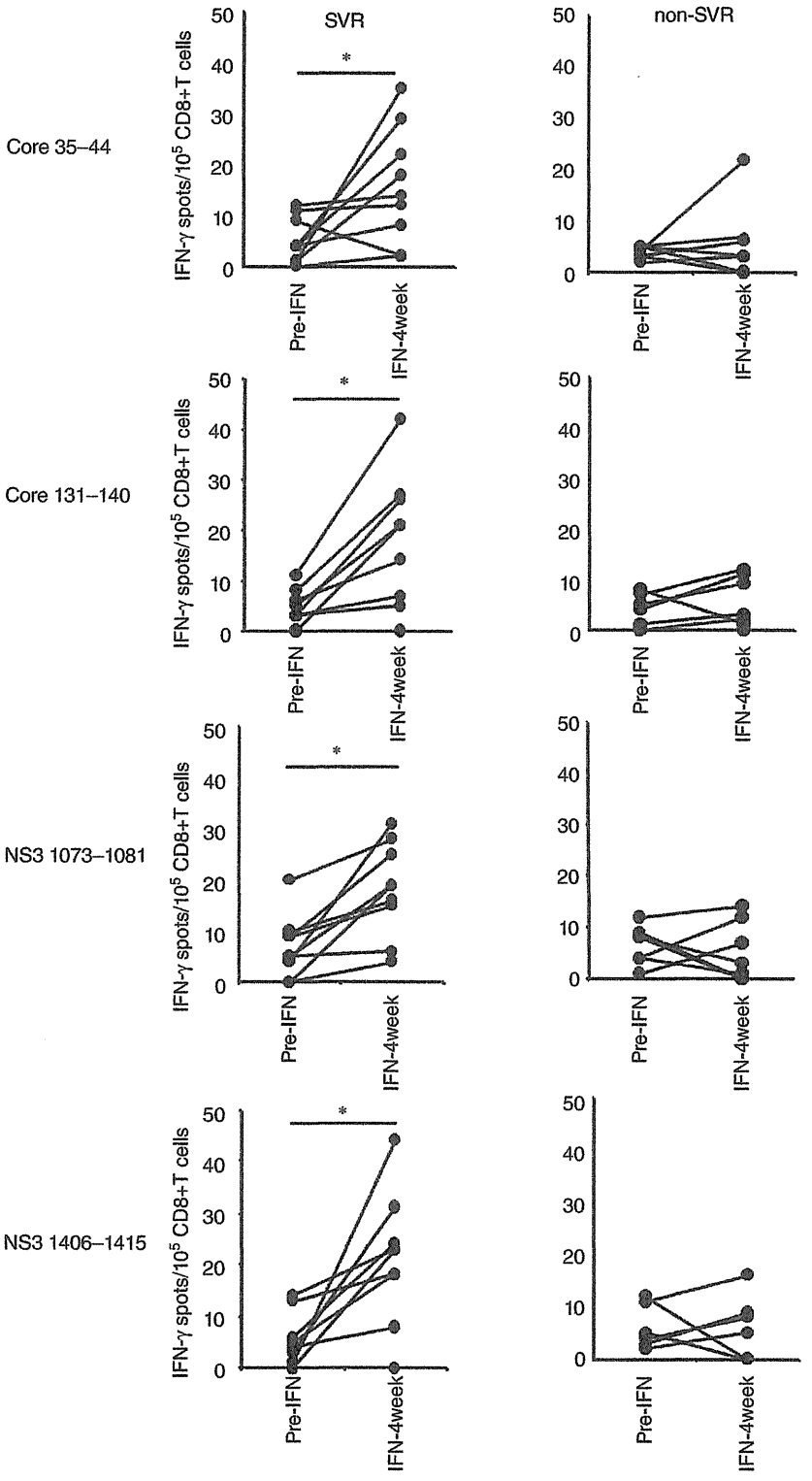

Figure 4 Analysis of the association of the change of hepatitis C virus (HCV)-specific CD8+ T cell responses between pre-IFN and IFN-4week chronic hepatitis C (CH-C) patients with the achieving sustained virologic response (SVR). Peripheral blood CD8+ T cells were isolated from pre-IFN and IFN-4week patients. HCV-specific CD8+ T cell responses were evaluated by interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assay. We analyzed the association of HCV-specific CD8+ T cell responses in treated CH-C patients with the achieving SVR. Each symbol within a panel represents the response of an individual donor to the indicated HLA-A2-presenting HCV Core or NS3 protein-derived peptides. The treated patients were divided into two groups; SVR group and non-SVR group. $^*P < 0.05$.

pre-terminally differentiated CD8+ T cells was also observed in non-SVR patients.[16] These results suggested that CTLs maturation efficiently occurred in SVR patients. HCV or HCV-gene products have been reported to inhibit the maturation pathway of CTLs.[5,21] Thus the decrease of viral load during this combination therapy may induce CTL maturation.

We demonstrated that the achievement of SVR in this combination therapy was associated with the early elevation of HCV-specific CD8+ T cell responses, but not with the pre-treated levels of HCV-specific CD8+ T cell responses. These results suggested, at least, that the enhancement of HCV-specific CD8+ T cell responses might play critical roles in the second slope of viral clearance by this combination therapy. The increasing frequencies of HCV-specific CD8+ T cells have also been reported to be associated with SVR during the combination therapy by evaluating with pentamers of HCV-specific peptides.[16] Ribavirin has immunomodulatory effect with a switch from Th2 to Th1 cytokine profile.[22] The combined use of pegIFNα and ribavirin might have more immunomodulatory effect to generate HCV specific CTLs. However, even now, this should be elucidated to develop better treatment of chronic hepatitis C.

Although CTL responses to HCV are multi-specific,[13,23] we and others tested only small part of the known CTL epitopes of HCV, which do not comprise all potential HLA A2-restricted CTL epitopes of HCV. HCV may have mutated and escaped from the CTL responses to the corresponding epitopes in the chronically infected patients. The epitopes used in our study have been applied to the detection of HCV-specific CTLs in several other previous studies,[5,15,16] which support the usefulness of the selected epitopes. Our results demonstrated that the increases of the frequencies of CD8+ T cells against four synthesized peptides were associated with the antiviral activity of this combination therapy. Thus the selected epitopes used in our experiments were probably stable, at least, during the 4 weeks after starting treatment.

In spite of recent progress for HCV treatment, there remains significant room for improvement. To date, a variety of viral factors and host factors that correlate with SVR in the combination therapy have been noted. Recently, in addition to viral factors and host factors, response and adherence to treatment have been noted.[2] To establish the better treatment, the detail mechanism of HCV elimination should be elucidated. In the present study, we demonstrated that early enhancement of HCV-specific CD8+ T cell responses was associated with the achieving SVR in this combination therapy. These suggest that activation of antiviral CTLs might be involved in the elimination of HCV. The early elevation of HCV-specific CTL responses in treated HCV patients may be a candidate for predicting SVR in this combination therapy.

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Letters in Applied Microbiology

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NOTE TO THE EDITOR

Evaluation of a chromogenic agar medium for the detection of extended-spectrum β-lactamase-producing *Enterobacteriaceae*

R. Saito[1,2], S. Koyano[1,2], R. Nagai[1,2], N. Okamura[2], K. Moriya[1] and K. Koike[1]

1 Department of Infection Control and Prevention, The University of Tokyo Hospital, Tokyo, Japan
2 Department of Microbiology and Immunology, Graduate School of Health Sciences, Tokyo Medical and Dental University, Tokyo, Japan

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AmpC β-lactamases, chromogenic media, CTX-M, *Enterobacteriaceae*, extended-spectrum β-lactamases.

Correspondence
Ryoichi Saito, Department of Infection Control and Prevention, The University of Tokyo Hospital, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.
E-mail: saito-lab@umin.ac.jp.

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Abstract

Aim: To compare the performance of a new chromogenic agar medium CHROMagar ESBL (KC-ESBL) to chromID ESBL (SB-ESBL) for the detection and presumptive identification of extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* directly from clinical specimens.

Methods and Results: A total of 256 specimens were screened for ESBL producers. Also, the genotypes of the ESBLs and plasmid-mediated AmpC β-lactamases (pAmpCBLs) were characterized by PCR and sequencing. Among the 256 specimens, 17 (6·6%) ESBL producers were isolated on both media. The sensitivity, specificity, positive predictive value and negative predictive value were higher for KC-ESBL (100, 93·3, 51·5 and 100%, respectively) than for SB-ESBL (88·2, 92·9, 46·9 and 99·1%, respectively) ($P = 0·72$). *Enterobacteriaceae* harbouring pAmpCBL genes as well as chromosomal cephalosporinase- and penicillinase-hyperproducing *Enterobacteriaceae* and *Pseudomonas aeruginosa* accounted for the false-positive results.

Conclusion: KC-ESBL can detect ESBL producers from clinical specimens with good selectivity and rapid presumptive identification by means of colony colour at 24 h.

Significance and Impact of the Study: This is the first study that has evaluated the performance of KC-ESBL that enables the detection and presumptive identification of ESBL producers from clinical specimens.

The proliferation of clinical isolates producing β-lactam-hydrolysing enzymes, such as extended-spectrum β-lactamases (ESBLs), has become a great concern. Because infectious diseases caused by strains producing these enzymes are associated with severe adverse clinical outcomes, it is essential to have rapid diagnostic methods for the detection of ESBL-producing organisms from clinical specimens. Recently, a chromogenic agar medium chromID ESBL (SB-ESBL; Sysmex-bioMerieux, Tokyo, Japan) has become commercially available for the rapid detection of ESBL producers (Glupczynski *et al.* 2007; Reglier-Poupet *et al.* 2008). This study compared the performance of a new chromogenic agar medium CHROMagar ESBL (KC-ESBL; Kanto Chemical, Tokyo, Japan) to SB-ESBL in the detection and presumptive identification of ESBL-producing *Enterobacteriaceae* from clinical specimens.

Our samples were a total of 256 nonduplicate specimens referred to our laboratory for the screening of ESBL-producing organisms. These specimens were collected from hospitalized patients between June 2008 and August 2008 and came from the following sites: 186 stool, 48 urine, 12 sputum, and 10 wound swabs. Each stool, sputum and wound swab specimen was homogenized in 0·5 ml of sterile physiological saline. Then, using calibrated loops, 10 μl of the resulting suspension was inoculated onto SB-ESBL and KC-ESBL. For urine samples, 10 μl of the sample was plated directly onto both media. SB-ESBL was obtained from the manufacturer as a prepared plate medium, and

KC-ESBL was prepared from a dehydrated medium according to manufacturer's instructions. All media were incubated at 35°C under aerobic conditions and assessed after 24 h of incubation. Reference strains of *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as the negative and positive control for ESBL production, respectively. Ten clinical isolates of ESBL-producing *E. coli* (three CTX-M-type and one SHV-type), *Kl. pneumoniae* (one CTX-M-type and two SHV-types) and *Proteus mirabilis* (three CTX-M-type) were also tested for their ability to grow on both media using the modified Miles–Misra method (Miles *et al.* 1938). The colour and intensity of the colonies on both media was recorded according to the colouration types provided by the manufacturer's instructions. All isolates growing on SB-ESBL and/or KC-ESBL were regarded as presumptive ESBL producers and were identified by the Microscan Walkaway 96 system (Siemens Healthcare Diagnostics Inc., Tokyo, Japan). For the confirmation of ESBL-positive isolates, a synergy test was performed and interpreted according to CLSI guidelines (CLSI 2008). Furthermore, ESBL producers were confirmed by using a double-disc diffusion (DDD) method described by Wiegand *et al.* (2007). Genotypes of bla_{TEM}, bla_{SHV} and bla_{CTX-M} and plasmid-mediated AmpC β-lactamases (pAmpCBLs) were characterized according to published methods (Yagi *et al.* 2000; Perez-Perez and Hanson 2002; Shibata *et al.* 2006). The constitutive overexpression of chromosomal cephalosporinases and penicillinases was determined as described by Livermore *et al.* (2007). The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were assessed by the chi-square test using JMP software, version 6.0 (SAS Institute, Tokyo, Japan). A two-tailed *P* value of <0.05 was taken as significant.

The two media gave a similar isolation rate in ten clinical isolates of ESBL producers, and all three methods (a synergy test, a DDD method and the molecular techniques) showed similar sensitivities for the detection of ESBL producers in all strains growing on the both media (data not shown). From the 256 specimens, 17 (6·6%) ESBL-producing *Enterobacteriaceae* were isolated on KC-ESBL (Table 1). Although, for SB-ESBL, among the 17 isolates, two strains of ESBL-producing *E. coli* failed to produce a chromogenic reaction, with no colour evident at 24 h, the use of a spot indole test and oxidase test on the colourless colonies allowed a rapid presumptive identification of *E. coli*. This result was similar to the previous finding (Reglier-Poupet *et al.* 2008) and was thought to be because of the use of different chromogenic substrates in each of the two media to detect specific bacterial enzymes, such as β-glucuronidase and β-galactosidase.

The sensitivity, specificity, PPV and NPV at 24 h were higher for KC-ESBL (100, 93·3, 51·5 and 100%, respectively) than for SB-ESBL (88·2, 92·9, 46·9 and 99·1%, respectively). However, these respective differences were not significant ($P = 0·72$). While the sensitivity of KC-ESBL was higher than that reported by Reglier-Poupet *et al.* (2008) and Glupczynski *et al.* (2007), the specificity of KC-ESBL was lower than that reported by Reglier-Poupet *et al.* (2008) but higher than that reported by Glupczynski *et al.* (2007). The PPV of KC-ESBL was higher than that of SB-ESBL and that reported by Reglier-Poupet *et al.* (2008). The high NPV on both media also permitted a quick and easy confirmation of the absence of ESBL-producing *Enterobacteriaceae* in a clinical sample as described previously (Reglier-Poupet *et al.* 2008).

Regarding false-positive strains (ESBL-negative organisms on the disc synergy testing, the DDD method and PCR) growing on each medium with the correct enterobacterial species chromogenic character, 22 were observed on SB-ESBL and 21 on KC-ESBL. In these strains, pAmpCBL genes were detected in seven *Enterobacter cloacae*,

Table 1 Characterization of extended-spectrum β-lactamase producers detected on either of two media

Species (no. of isolates)	Molecular typing of β-lactamases			
	CTX-M group	SHV	TEM	pAmpCBL
Escherichia coli (1)	CTX-M-1	–	–	–
E. coli (2)	CTX-M-2	–	–	–
E. coli (3)	CTX-M-9	–	–	–
E. coli (2)	CTX-M-1	–	TEM-1	–
E. coli (1)	CTX-M-1	–	TEM-1	MOX
E. coli (1)	CTX-M-2	–	TEM-1	MOX
E. coli (1)	–	SHV-12	–	–
E. coli (1)	–	SHV-12	TEM-1	–
Klebsiella pneumoniae (2)	–	SHV-12	–	–
Klebsiella oxytoca (1)	CTX-M-9	–	–	–
Enterobacter cloacae (1)	CTX-M-9	SHV-12	–	EBC
Citrobacter freundii (1)	CTX-M-9	–	–	–

pAmpCBL, plasmid-mediated AmpC β-lactamase.

one *Enterobacter aerogenes* and three *Citrobacter freundii*. Chromosomal cephalosporinase-hyperproducing isolates of three *Ent. cloacae* and two *Cit. freundii*, and one chromosomal penicillinase-hyperproducing *Klebsiella oxytoca* were also observed. This result was in agreement with previous studies (Glupczynski *et al.* 2007; Reglier-Poupet *et al.* 2008) and might be accomplished by the incorporation of improved inhibitors of AmpC β-lactamase, such as phenylboronic acid. Five non-*Enterobacteriaceae* isolates of *Pseudomonas aeruginosa* grown on SB-ESBL (four *Ps. aeruginosa* on KC-ESBL), which produced a green or brown colour, also accounted for false-positive results as described previously (Reglier-Poupet *et al.* 2008). However, these isolates were easily identified on the both media by the pattern of the colonies and/or by a rapid simple test (positive oxidase reaction) which could be performed directly on the colonies.

In conclusion, the present study has a concern limitation, in that the numbers of ESBL producer detected were small. However, KC-ESBL can detect most ESBL producers with good selectivity and reduce workload for the screening of those as well as SB-ESBL.

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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

Hideyuki Miyoshi[1], Kyoji Moriya[1], Takeya Tsutsumi[1], Seiko Shinzawa[1], Hajime Fujie[1], Yoshizumi Shintani[1], Hidetake Fujinaga[1], Koji Goto[1], Toru Todoroki[2], Tetsuro Suzuki[3], Tatsuo Miyamura[3], Yoshiharu Matsuura[4], Hiroshi Yotsuyanagi[1], Kazuhiko Koike[1,*]

[1]*Department of Internal Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan;* [2]*Department of Laboratory Medicine, Keio University School of Medicine, Tokyo, Japan;* [3]*Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan;* [4]*Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan*

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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* Corresponding author. Address: Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Tel.: +81 3 5800 8800; fax: +81 3 5800 8799.

E-mail address: kkoike-tky@umin.ac.jp (K. Koike).

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.

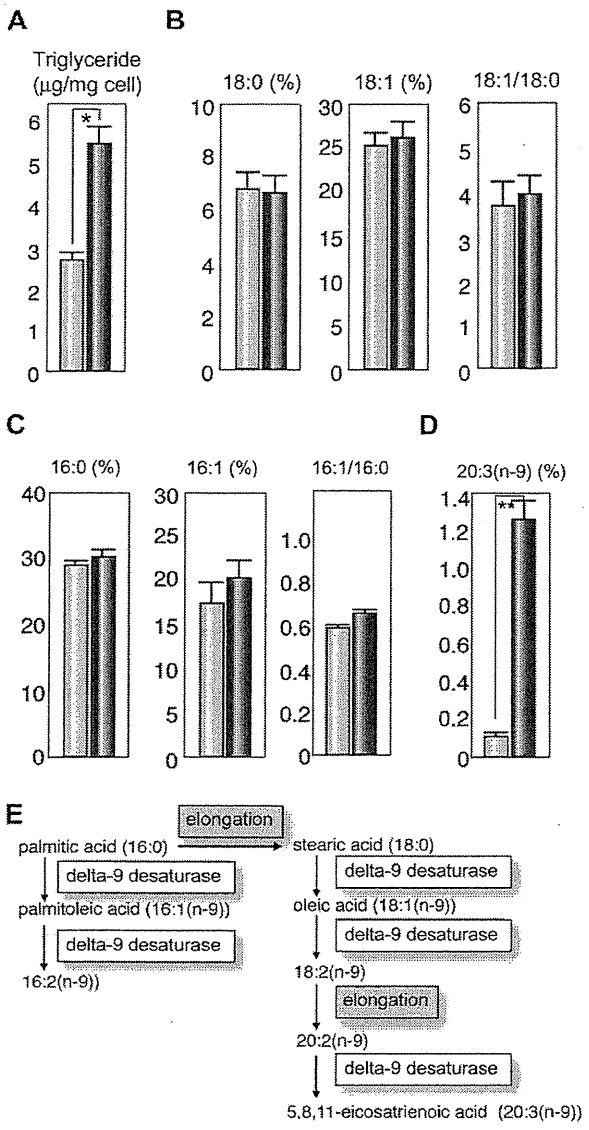

Fig. 1. Effect of the core protein on fatty acid composition in HepG2 cells. The fatty acid compositions of the total cell lipids were analyzed and the ratios of 18:1/18:0 and 16:1/16:0 in the core-expressing and control HepG2 cells were calculated. (A) Concentrations of triglycerides. (B) Percentages 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.

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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

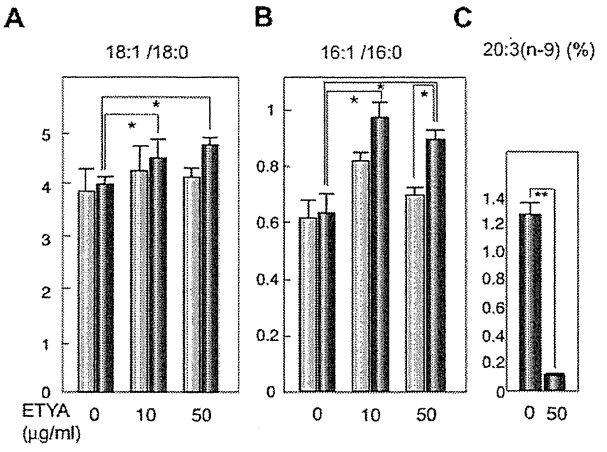

Fig. 2. Effect of ETYA on delta-9 desaturase index. HepG2 cells with or without the core protein were incubated with ETYA for 48 h. The fatty acid compositions of the total cell lipids were analyzed, and the ratios of 18:1/18:0 (A) and 16:1/16:0 (B), and the percentage of eicosatrienoic acid (20:3(n-9) (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

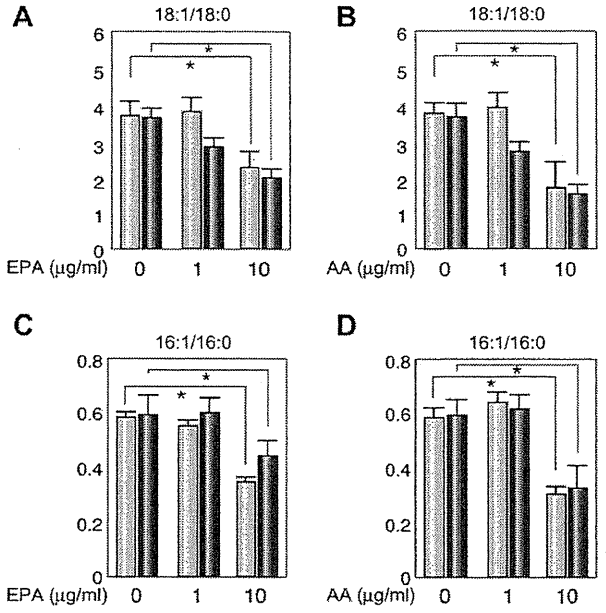

Fig. 3. Effect of EPA and AA on delta-9 desaturase index. HepG2 cells with or without the core protein were incubated with EPA (A and C) or AA (B and D) for 48 h. The fatty acid compositions of the total cell lipids were analyzed and the ratios of 18:1/18:0 (A and B) and 16:1/16:0 (C and D) were computed. 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.

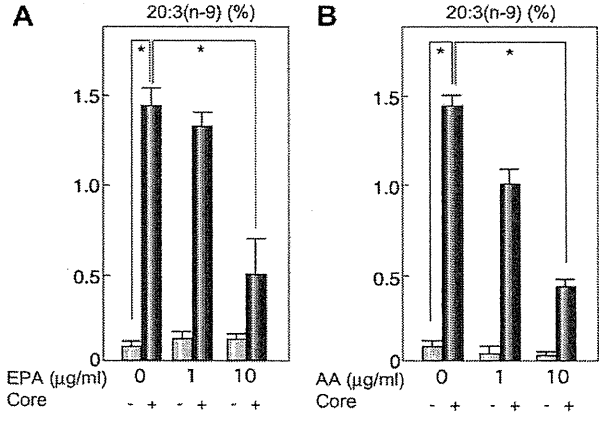

Fig. 4. Effect of EPA and AA on the concentration of 20:3(n-9). HepG2 cells with or without the core protein were incubated with EPA (A) or AA (B) for 48 h. The fatty acid compositions of the total cell lipids were analyzed and the percentages of the C20:3(n-9) fraction were measured. Light blue bars 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.

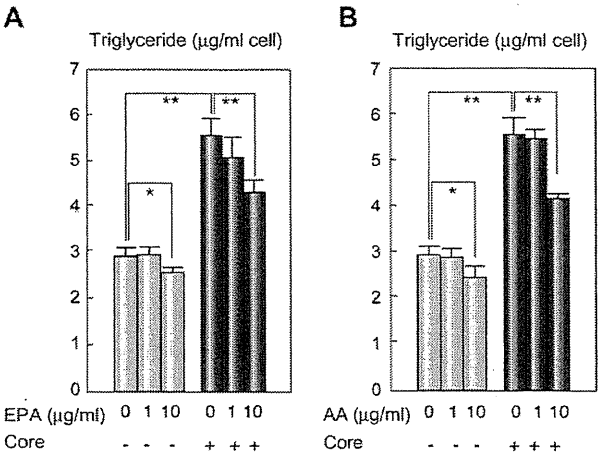

Fig. 5. Effect of EPA and AA on triglyceride content. HepG2 cells with or without the core protein were incubated with EPA (A) or AA (B) for 48 h. The triglyceride volume of the total cell lipids was measured and the triglyceride contents in the cells were calculated. Light blue bars indicate control 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.

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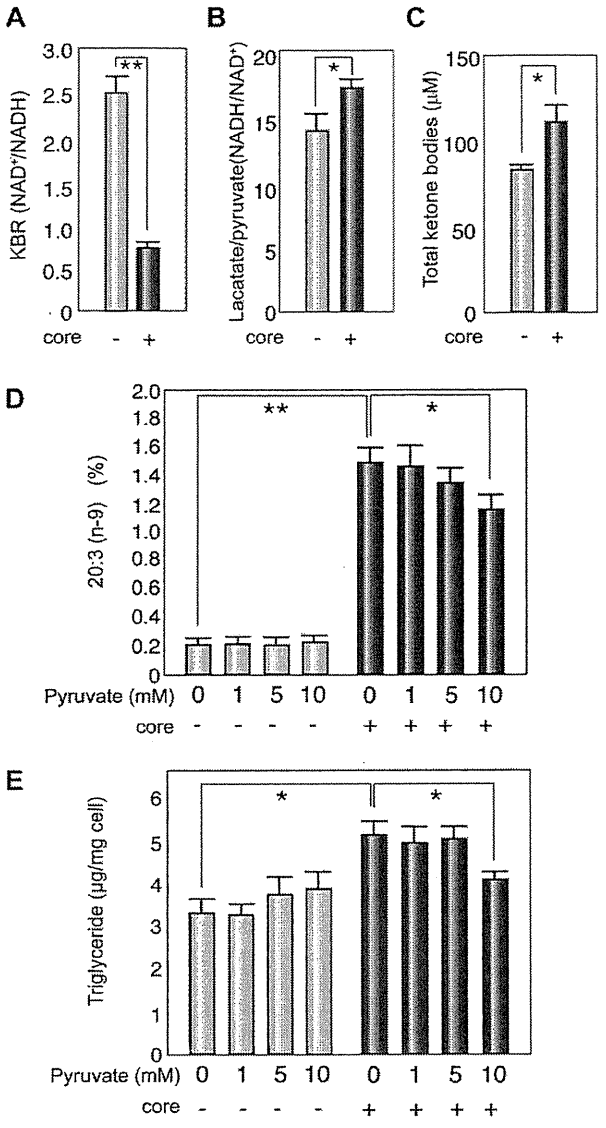

Fig. 6. NADH accumulation and effect of pyruvate in core-expressing cells. HepG2 cells with or without the core protein were subjected to the determination of ketone body ratio (A) and lactate/pyruvate ratio (B) for the precise estimation of NAD^+/NADH and NADH/NAD^+. (C) Total ketone bodies. (D) The 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

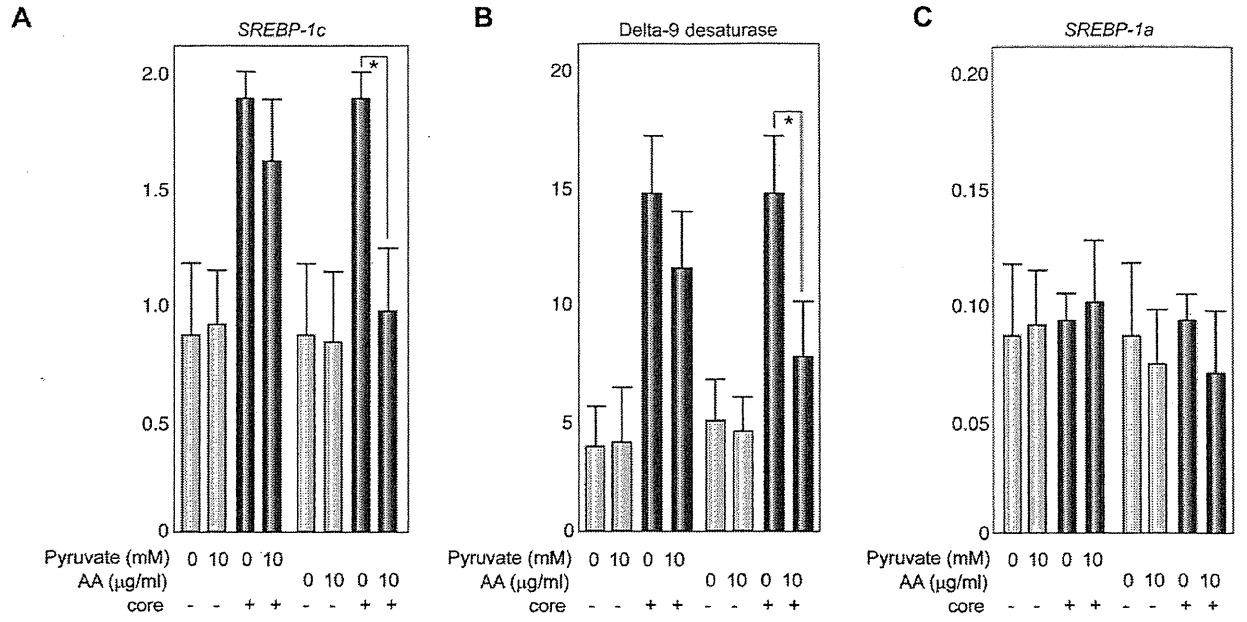

Fig. 7. Effect of pyruvate and AA on mRNA levels of lipid-associated genes. The mRNA levels of *SREBP-1c* (A), delta-9 desaturase (B) and *SREBP-1a* (C) genes were determined by real-time PCR analysis. The transcription of the genes was normalized with that of hypoxanthine phosphoribosyltransferase, 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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Lipid Metabolism and Liver Disease in Hepatitis C Viral Infection

Kazuhiko Koike Takeya Tsutsumi Hiroshi Yotsuyanagi Kyoji Moriya

Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

Key Words

Hepatitis C · Hepatocellular carcinoma · Transgenic mouse · Core protein · Steatosis · Insulin resistance · Oxidative stress

Abstract

Persistent infection with hepatitis C virus (HCV) is a major risk toward development of hepatocellular carcinoma. A number of transgenic mouse lines carrying the cDNA of HCV genome have been established and evaluated in the study of HCV pathogenesis. Among those, the studies using transgenic mouse lines that carry the HCV genome containing the core gene indicate the direct involvement of HCV in pathogenicity, including that in oncogenesis. Oxidative stress overproduction and intracellular signaling augmentation are shown to be the key events in HCV-associated hepatocarcinogenesis. Besides the data in hepatitis C patients, connecting liver fibrosis progression and the disturbance in lipid and glucose metabolisms, these mouse models also show a close relationship between HCV and metabolic alterations including hepatic steatosis and insulin resistance. Furthermore, the persistent activation of peroxisome proliferator-activated receptor-α has recently been found, yielding dramatic changes in the lipid metabolism and oxidative stress overproduction in cooperation with the mitochondrial dysfunction. These results would provide a clue for further understanding of the role of lipid metabolism in pathogenesis of hepatitis C including liver injury and hepatocarcinogenesis.

Introduction

Hepatitis C virus (HCV) infection frequently evolves into a persistent state, leading to the development of chronic hepatitis, cirrhosis, and, eventually, hepatocellular carcinoma (HCC). Recently, there have been increasing lines of evidence to indicate metabolic disturbances in HCV infection, which would influence the pathogenesis of chronic hepatitis C. The discovery of HCV in 1989 enabled the comparison between chronic hepatitis C and the other chronic hepatitis, resulting in repeated reports that steatosis is significantly associated with chronic hepatitis C [1, 2]. Steatosis in HCV infection is reproduced in animal models [3] or cultured cells [4], strengthening a pathologic role of HCV in it. Furthermore, patients infected with HCV have abnormalities in serum lipids, such as hypocholesterolemia or abnormal levels of apolipoproteins in serum [5, 6]; they are corrected in sustained virological responders to antiviral treatment [6]. Thus, the association between HCV infection and disturbance in lipid metabolism has become increasingly strong both in patients and experimental systems including animals. Finally, patients with chronic hepatitis C accompanied by severe steatosis develop hepatic fibrosis more rapidly [7]. Thus, abnormal lipid metabolism in HCV infection would be deeply involved in the pathogenesis of hepatitis C.

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Kazuhiko Koike, MD, PhD
Department of Gastroenterology
Graduate School of Medicine, University of Tokyo
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655 (Japan)
Tel. +81 3 5800 8800, Fax +81 3 5800 8799, E-Mail kkoike-tky@umin.ac.jp

HCV and Hepatocarcinogenesis

The mechanism underlying hepatocarcinogenesis in HCV infection is not fully understood yet, despite the fact that nearly 80% of patients with HCC in Japan are persistently infected with HCV [8]. HCV infection is also common in patients with HCC in other countries, albeit to a lesser extent. These lines of evidence prompted us to seek for determining the role of HCV in hepatocarcinogenesis. Inflammation induced by HCV should be considered, of course, in a study on the hepatocarcinogenesis in hepatitis viral infection: necrosis of hepatocytes due to chronic inflammation followed by regeneration enhances genetic aberrations in host cells, the accumulation of which culminates in HCC. This theory presupposes an indirect involvement of hepatitis viruses in HCC via hepatic inflammation. However, this context leaves us with a serious question: Can inflammation alone result in the development of HCC in such a high incidence (90% in 15 years) or multicentric nature in HCV infection?

The other role of HCV would have to be weighed against an extremely rare occurrence of HCC in patients with autoimmune hepatitis in which severe inflammation in the liver persists indefinitely, even after the development of cirrhosis. These backgrounds and reasonings lead to a possible activity of viral proteins for inducing neoplasia. This possibility has been evaluated by introducing genes of HCV into hepatocytes in culture with little success. One of the difficulties in using cultured cells is the carcinogenic capacity of HCV, if any, which would be weak and would take a long time to manifest itself. Actually, it takes 30–40 years for HCC to develop in individuals infected with HCV. On the basis of these viewpoints, we started to investigate carcinogenesis in chronic hepatitis C, in vivo, by transgenic mouse technology.

Transgenic Mouse Lines Carrying the HCV Genome

As described above, the HCV proteins have been characterized chiefly using in vitro translation or cultured cells. Little is known, however, about the role of HCV or its proteins in the pathogenesis of hepatitis and subsequent liver diseases, cirrhosis and HCC. One of the major issues regarding the pathogenesis of HCV-associated liver lesion is whether the HCV proteins have direct effects on pathological phenotypes. Although several strategies have been used to characterize the hepatitis C viral proteins, the relationship between the protein expression and disease phenotype has not been clarified. For this purpose, several lines of mice have been established which were transgenic for the HCV cDNA. They include the ones carrying the entire coding region of HCV genome [9], the core region only [3, 10], the envelope region only [11], the core and envelope regions [12] and the core to non-structural (NS)2 regions [13]. Although detection of mRNA from the NS regions of the HCV cDNA has been reported [9], the detection of HCV NS proteins in the transgenic mouse liver has not been successful. The reason for this failure in detecting NS proteins is unclear, but the expression of the NS enzymes may be harmful to mouse development and may allow the establishment of only low-expression mice.

In terms of expression system, two different ways have been applied: transient and constitutive expression systems. One transgenic mouse line has been reported which expresses the HCV genes using a transient expression system. Wakita et al. [13] utilized the Cre/*lox*P system, by which a gene under silent can be switched on by the introduction of Cre recombinase. They established a transgenic mouse line that had the core, envelopes and NS2 genes of HCV in a silent state. After the injection of the recombinant adenovirus that had *Cre* recombinase in the mice, the HCV genes expressed transiently. These mice developed acute hepatitis, which was blocked by the administration of anti-CD4 and CD8 antibodies. This mouse system would provide a good animal model for acute hepatitis C and be useful for the study of immunological aspects of hepatitis. The possibility, however, that the greatly overexpressed HCV proteins had caused the death of hepatocytes and provoked the immune response thereafter still remains.

We have engineered transgenic mouse lines carrying the HCV genome were by introducing the genes from the cDNA of the HCV genome of genotype 1b [3, 14]. Established are four different kinds of transgenic mouse lines, which carry the core gene, envelope genes, the entire NS genes, or NS5A gene, respectively, under the same transcriptional regulatory element (fig. 1). Among these mouse lines, only the transgenic mice carrying the core gene developed HCC in two independent lineages [14]. The envelope gene transgenic mice do not develop HCC, despite high expression levels of both E1 and E2 proteins [11, 15], and the transgenic mice carrying the entire NS or NS5A gene have developed no HCC.

The core gene transgenic mice express the core protein of an expected size, and the level of the core protein in the liver is similar to that in chronic hepatitis C patients. Early in life, these mice develop hepatic steatosis, which is

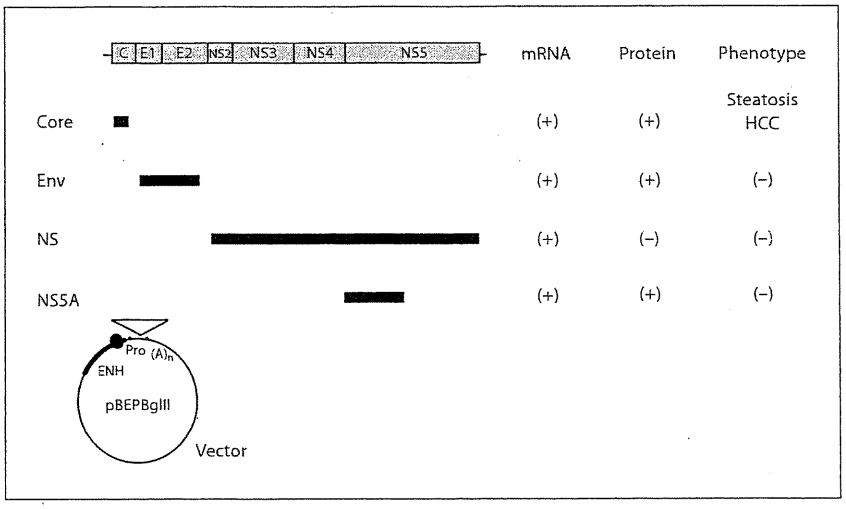

Fig. 1. Transgenic mouse lines carrying the HCV genome. Four different kinds of transgenic mouse lines, carrying the core gene, envelope genes, the entire NS genes, or NS5A of HCV, respectively, were established under the control of the same regulatory elements. Among these mouse strains, only the transgenic mice carrying the HCV core gene develop HCC after an early phase with hepatic steatosis in two independent lineages. HCV = Hepatitis C virus; HCC = hepatocellular carcinoma; Env = envelope genes; NS = non-structural genes.

one of the histologic characteristics of chronic hepatitis C, along with lymphoid follicle formation and bile duct damages [1]. Thus, the core gene transgenic mouse model well reproduces the feature of chronic hepatitis C. Of note, any pictures of significant inflammation are not observed in the liver of this animal model. Late in life, these transgenic mice develop HCC. Notably, the development of steatosis and HCC has been reproduced by other HCV transgenic mouse lines, which harbor the entire HCV genome or structural genes including the core gene [9, 10, 12]. These outcomes indicate that the core protein per se of HCV has an oncogenic potential when expressed in vivo.

Enhancement of Oxidative Stress and Intracellular Signaling in HCV-Associated Hepatocarcinogenesis

It is difficult to elucidate the mechanism underlying the development of HCC, even for our simple model in which only the core protein is expressed in otherwise normal liver. There is a notable feature in the localization of the core protein in hepatocytes; while the core protein predominantly exists in the cytoplasm associated with lipid droplets, it is also present in the mitochondria and nuclei [14]. On the basis of this finding, the pathways related to these two organelles, the mitochondria and nuclei, were thoroughly investigated.

One effect of the core protein is an increased production of oxidative stress in the liver. We would like to draw particular attention to the fact that the production of oxidative stress is increased in our transgenic mouse model in the absence of inflammation in the liver. This reflects a state of an overproduction of reactive oxygen species (ROS) in the liver, or predisposition to it, which is staged by the HCV core protein without any intervening inflammation [16, 17]. The overproduction of oxidative stress results in the generation of deletions in the mitochondrial and nuclear DNA, an indicator of genetic damage. In addition, analysis of antioxidant system revealed that some antioxidative molecules are not increased despite the overproduction of ROS in the liver of core gene transgenic mice. These results suggest that HCV core protein not only induces overproduction of ROS but also attenuates some of antioxidant system, which may explain the mechanism underlying the production of a strong oxidative stress in HCV infection compared to other forms of hepatitis.

In the absence of inflammation, thus, the core protein induces oxidative stress overproduction, which may, at least in part, contribute to hepatocarcinogenesis in HCV infection. If inflammation were added to the liver with the HCV core protein, the production of oxidative stress would be escalated to an extent that cannot be scavenged anymore by a physiological antagonistic system. This suggests that the inflammation in chronic HCV infection would have a characteristic different in its quality from those of other types of hepatitis, such as autoimmune hepatitis. The basis for the overproduction of oxidative stress may be ascribed to the mitochondrial dysfunction [16, 18]. The dysfunction of the electron transfer system of the mitochondrion is suggested in as-

sociation with the presence of the HCV core protein [18, 19].

Other pathways in hepatocarcinogenesis would be the alteration of the expression of cellular genes and modulation of intracellular signaling pathways. For example, tumor necrosis factor (TNF)-α and interleukin-1β have been found transcriptionally activated [20]. The mitogen-activated protein kinase (MAPK) cascade is also activated in the liver of the core gene transgenic mouse model. The MAPK pathway, which consists of three routes, c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK), is involved in numerous cellular events including cell proliferation. In the liver of the core gene transgenic mouse model prior to HCC development, only the JNK route is activated. In the downstream of the JNK activation, transcription factor activating protein (AP)-1 activation is markedly enhanced [20, 21]. At far downstream, both the mRNA and protein levels of cyclin D1 and cyclin-dependent kinase (CDK)4 are increased. Thus, the HCV core protein modulates the intracellular signaling pathways and gives advantage for cell proliferation to hepatocytes.

Hepatitis C as a Metabolic Disease

Steatosis is frequently observed in chronic hepatitis C patients, and significantly associated with increased fibrosis and progression rate of fibrosis of the liver [22]. A comprehensive analysis of gene expression in the liver of core gene transgenic mice, in which steatosis develops from early in life, revealed that a number of genes related to lipid metabolism are significantly up- or downregulated.

The composition of fatty acids that are accumulated in the liver of core gene transgenic mice is different from that in fatty liver due to simple obesity. Carbon-18 monounsaturated fatty acids (C18:1) such as oleic or vaccenic acids are significantly increased. This is also the case in the comparison of liver tissues from hepatitis C patients and simple fatty liver patients due to obesity [17]. The mechanism of steatogenesis in hepatitis C was investigated using this mouse model. There are, at least, three pathways for the development of steatosis. One is the frequent presence of insulin resistance in hepatitis C patients as well as in the core gene transgenic mice, which occurs through the inhibition of tyrosine phosphorylation of insulin receptor substrate (IRS)-1 [23]. Insulin resistance increases the peripheral release and hepatic uptake of fatty acids, resulting in an accumulation of lipid in the liver. The second pathway is the suppression of the activity of microsomal triglyceride transfer protein (MTP) by HCV core protein [24]. This inhibits the secretion of very-low-density protein (VLDL) from the liver, yielding an increase of triglycerides in the liver. The last one involves by the sterol regulatory element-binding protein (SREBP)-1c, which regulates the production of triglycerides and phospholipids. In HCV core gene transgenic mice, SREBP-1c is activated, while neither SREBP-2 nor SREBP-1a is upregulated [25].

Steatosis, HCV and PA28γ

Interestingly, we found recently that a protein interacting with the core protein, proteasome activator (PA)28γ, is indispensable for the core protein to exert its function for the development of steatosis, insulin resistance and HCC [3, 25]. Steatosis is defined as an accumulation of lipid droplets, a majority of which are triglycerides. Biosynthesis of triglycerides is mainly regulated by SREBP-1c. Transcription of SREBP-1c is controlled by a heterodimer of nuclear hormone receptors, liver X receptor (LXR)-α and retinoid X receptor (RXR)-α. Indeed, it has been reported that many genes regulated by SREBPs were induced during the early stage of HCV infection in the livers of chimpanzees. Our study has demonstrated that the core protein enhances the binding activity of the LXR-α–RXR-α complex to the *srebp-1c* promoter in a PA28γ-dependent manner, resulting in upregulation of SREBP-1c and its regulating genes [25]. The activation may be mediated by the direct interaction between the core protein and RXR-α [26] or by suppression of a corepressor such as Sp110b, a negative regulator of RAR-α, by sequestering it in the cytoplasm via interaction with the cytoplasmic core protein [27]. Another mechanism is thought to be suppression of lipid secretion. Reduced serum levels of cholesterol and apolipoprotein B have been reported in patients with severe hepatitis C and the core gene transgenic mice [5]. As stated before, the MTP regulates the assembly and secretion of VLDLs consisting of apolipoprotein B, cholesterol and triglycerides. In the core gene transgenic mice, MTP-specific activity is significantly decreased. Therefore, the downregulation of MTP may be involved in the development of the steatosis cooperating with upregulation of SREBP-1c, although the precise role of HCV core protein is still unclear. Recently, it has been reported that the assembly and budding of HCV occur around the accumulated lipid droplets within the endoplasmic reticulum [28]. Furthermore,

increases in saturated and monounsaturated fatty acids enhance HCV RNA replication. These data suggest that regulation of lipid metabolism by the core protein plays crucial roles in the HCV life cycle. Obesity and hepatic steatosis often result in insulin resistance. However, 1- to 2-month-old core gene transgenic mice, which do not exhibit apparent steatosis and obesity, already exhibit insulin resistance due to a decrease in insulin sensitivity in the liver [23]. Moreover, the core gene transgenic mice have been shown to exhibit overt diabetes when fed a high-fat diet, while control mice do not. Binding of insulin to the insulin receptor triggers tyrosine phosphorylation of the IRS proteins, leading to the following signal transductions to increase glucose uptake and inhibit the net production of glucose in the liver. An inflammatory cytokine, TNF-α, is known to impair the insulin-signaling pathway via inhibition of tyrosine phosphorylation of IRSs. In fact, the overproduction of TNF-α has been reported to reduce the phosphorylation of IRS-1 and Akt in the core gene transgenic mice despite the absence of hepatic. In the latter study, moreover, hyperinsulinemia was cured by depletion of TNF-α, suggesting that upregulation of TNF-α contributes to the core protein-induced insulin resistance [23]. Our previous study has indicated that the core protein-induced overexpression of TNF-α is also dependent on the presence of PA28γ [25].

In relation to lipid metabolism, the core protein has also been found to interact with RXR-α [26]. RXR-α is one of the nuclear receptors which forms a homodimer or heterodimers with other nuclear receptors including peroxisome proliferator-activated receptor (PPAR)-α, and plays a pivotal role in the regulation of the expression of genes relating to lipid metabolism, cell differentiation and proliferation. In fact, the core protein of HCV activates genes that have an RXR-α-responsive element as well as those with a PPAR-α-responsive element, in both mice and cultured cells [26]. Based on these results, we, then, examined the expression and function of PPAR-α in the liver of core gene transgenic mice.

PPAR-α Activation and 'Fatty Acid Spiral' in HCV-Associated Hepatocarcinogenesis

PPAR-α is one of PPAR genes, and plays a central role, as a heterodimer with RXR-α, in regulating fatty acid transport and catabolism. It is also known as a molecular target for lipid-lowering fibrate drugs. On the other hand, a prolonged administration of PPAR-α agonists causes HCC in rodents. Currently, there is little evidence that the low-affinity fibrate ligands are associated with human cancers, but it is possible that chronic activation of high-affinity ligands could be carcinogenic in humans.

The level of PPAR-α protein was increased in the liver of core gene transgenic mice as early as 9 months. PPAR-α protein is accumulated with age in the nuclei of hepatocytes together with cyclin D1 protein. However, the level of PPAR-α mRNA was not increased at any age. By the pulse-chase experiment, the stability of nuclear PPAR-α turned out to be increased in the presence of the core protein. In line with the increase of PPAR-α protein, target genes of PPAR-α were activated in the liver of core gene transgenic mice; these genes include cyclin D1, CDK4, acy-CoA oxidase, and peroxisome thiolase [29]. However, in general, the activation of PPAR-α leads to improvement but not aggravation of steatosis. Then, what is a function of PPAR-α activation that is observed in the core gene transgenic mice?

To clarify the role of PPAR-α activation in pathogenesis of steatosis and HCC, we made mating of core gene transgenic mouse with PPAR-α knockout (KO) mouse, and studied the phenotype. PPAR-α KO mice have reduced expressions of target genes of PPAR-α, and have mild steatosis in the liver as expected. It was unanticipated, however, that steatosis was absent in PPAR-α-null or -heterozygous core gene transgenic mice but present in PPAR-α-intact core gene transgenic mice at the age of 9 or 24 months [29]. 8-Hydroxydeoxyguanosine and peroxylipids, both of which are markers for oxidative stress, were decreased in PPAR-α KO core gene transgenic mice. Mitochondrial dysfunction in the core gene transgenic mice, which contributes to an overproduction of oxidative stress, was also improved in PPAR-α KO core gene transgenic mice.

Finally, PPAR-α KO core gene transgenic mice did not develop HCC at the age of 24 months, while about one-third of PPAR-α-intact core gene transgenic mice did. It should be noted that core gene transgenic mice that are heterozygous for PPAR-α gene did not develop HCC either [29]. When clofibrate, a peroxisome proliferator, was administered for 24 months to PPAR-α-heterozygous mice, either with or without the core gene, HCC developed in a higher rate in the core-gene (+) mice with a greater PPAR-α activation. It should be noted that steatosis was present only in core-gene (+) PPAR-α-heterozygous mice. In summary, steatosis and HCC developed in PPAR-α-intact but not in PPAR-α-heterozygous or PPAR-α-null core gene transgenic mice, indicating that not the presence but the persistent activation of PPAR-α would be important in hepatocarcinogenesis by HCV

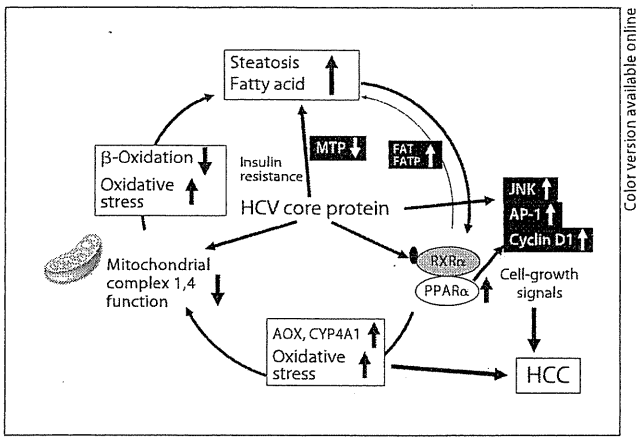

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Fig. 2. HCV core protein causes 'fatty acid spiral'. In HCV infection, the core protein induces steatosis via several pathways, leading to 'fatty acid spiral' in the presence of the mitochondrial complex-1 dysfunction and PPAR-α activation, both of which are also caused by the core protein. These intracellular alterations would contribute to hepatocarcinogenesis by inducing oxidative stress overproduction and cell-growth signal activation. In such a sense, the core protein of HCV is not a classical type oncoprotein, but rather seems to contribute to hepatocarcinogenesis by modulating intracellular metabolism and signaling. HCV = Hepatitis C virus; HCC = hepatocellular carcinoma; JNK = c-Jun N-terminal kinase; AP-1 = activating protein-1; RXRα = retinoid X receptor-α; PPARα = peroxisome proliferator activated receptor-α; AOX = acyl-CoA oxidase; CYP = cytochrome P450; MTP = microsomal triglyceride transfer protein; FAT = fatty acid translocase; FATP = fatty acid transport protein.

core protein. In general, PPAR-α acts to ameliorate steatosis, but with the presence of mitochondrial dysfunction, which is also provoked by the core protein, the core-activated PPAR-α may exacerbate steatosis. A persistent activation of PPAR-α with 'strong' ligands such as the core protein of HCV could be carcinogenic in humans, although the low-affinity fibrate ligands are not likely associated with human cancers.

Figure 2 illustrates our current hypothesis for the role of lipid metabolism in HCV-associated hepatocarcinogenesis. Immune-mediated inflammation should also play a pivotal role in hepatocarcinogenesis in HCV infection. However, in HCV infection, the core protein induces steatosis through the above-mentioned pathways, leading to the 'fatty acid spiral' in the presence of the mitochondrial electron transfer system dysfunction [18, 19] and PPAR-α activation, both of which are caused by the core protein. These intracellular alterations would contribute to hepatocarcinogenesis by inducing oxidative stress overproduction and cell-growth signal activation. In such a sense, the core protein of HCV is not a classical type oncoprotein, but rather seems to contribute to hepatocarcinogenesis by modulating intracellular metabolism and signaling.

Conclusion

The results of our studies on transgenic mice have indicated a carcinogenic potential of the HCV core protein in vivo; thus, HCV would be directly involved in hepatocarcinogenesis. In research studies of carcinogenesis, the theory by Kinzler and Vogelstein [30] has gained a wide popularity. They have proposed that the development of colorectal cancer is induced by the accumulation of a complete set of cellular gene mutations. They have deduced that mutations in the APC gene for inactivation, those in K-ras for activation and those in the p53 gene for inactivation accumulate, which cooperate toward the development of colorectal cancer [30]. Their theory has been extended to the carcinogenesis of other cancers as well, called 'Vogelstein-type' carcinogenesis.

On the basis of the results we obtained for the induction of HCC by the HCV core protein, we would like to introduce a different mechanism for the hepatocarcinogenesis in HCV infection. We do allow multistages in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that many mutations accumulate in hepatocytes. Some of these steps, however, may be skipped in the development of HCC in HCV infection to which the core protein would contribute. The overall effects achieved by the expression of the viral protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations, required for carcinogenesis.

By considering such a 'non-Vogelstein-type' process for the induction of HCC, a plausible explanation may be given for many unusual events happening in HCV carriers. Now it does not seem so difficult as before to determine why HCC develops in persistent HCV infection at an outstandingly high incidence. Our theory may also give an account of the non-metastatic and multicentric de novo occurrence characteristics of HCC, which would be the result of persistent HCV infection.

Disclosure Statement

The authors declare that they have no financial conflict of interest.

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