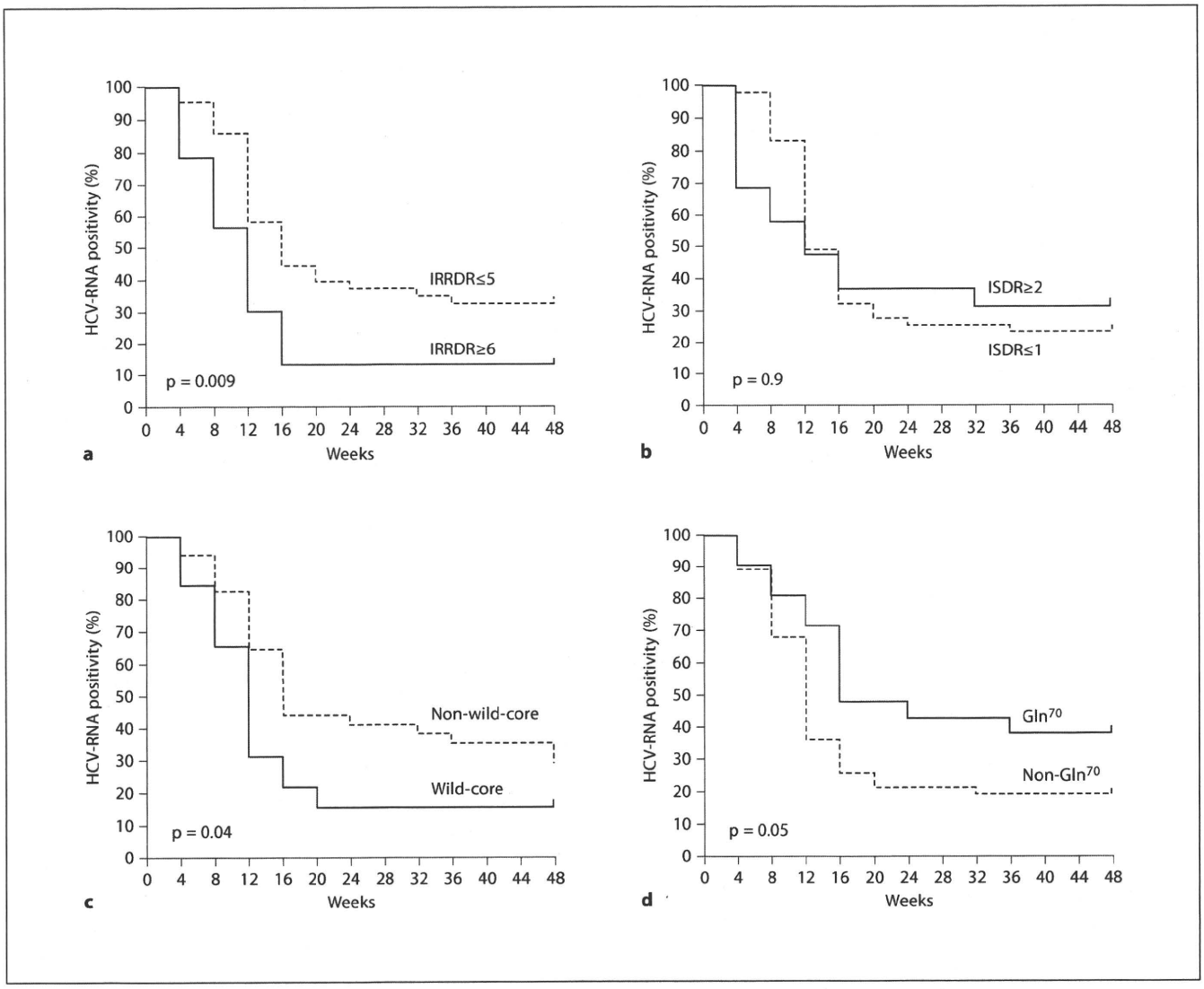

Fig. 2. Kaplan-Meier HCV survival curve analysis based on HCV-RNA positivity during the whole treatment course according to NS5A (**a**, **b**) and the core protein (**c**, **d**) polymorphisms. The difference between the analyzed groups was measured by the log-rank test.

polymorphisms. The result showed that HCV isolates of IRRDR≥6 were cleared from patients' sera more rapidly than those with IRRDR≤5 (fig. 2a). On the other hand, HCV-RNA clearance kinetics did not differ significantly between HCV isolates of ISDR≥2 and those of ISDR≤1 (fig. 2b). As for the core polymorphism, HCV isolates of non-wild-core or Gln^{70} persisted in patients' sera for longer periods of time than those of wild-core (Arg^{70}/Leu^{91}) or non-Gln^{70} (fig. 2c, d).

Next, HCV clearance kinetics during the very early stages of the treatment course, e.g., 24 h, 1, 2 and 4 weeks after initiation of PEG-IFN/RBV therapy was examined. For this purpose, a possible correlation between the degree of IRRDR, ISDR and core polymorphisms and the proportion of patients who achieved significant reduction (1 log after 24 h, 1 log after 1 week, 1.5 log after 2 weeks, and 2 log after 4 weeks) of core antigen titers was analyzed. Interestingly, IRRDR≥6 was significantly associated with reduction and/or disappearance of serum HCV core antigen titers at 24 h, 1, 2 and 4 weeks after initiation of the treatment (table 6). Again, there was no significant correlation between ISDR sequence variation

Table 6. Correlation between the proportions of patients with rapid reduction of HCV core antigen titers and degree of NS5A and core protein polymorphisms

Protein	Criteria	Number of patients with significant reduction of HCV core antigen titers/number of total							
		24 h[a] (≥1 log)[b]	p value	1 week (≥1 log)	p value	2 weeks (≥1.5 log)	p value	4 weeks (≥2 log)	p value
NS5A	IRRDR≥6	20/23	0.0006	18/23	0.004	17/23	0.018	19/23	0.008
	IRRDR≤5	17/40		16/40		16/40		19/40	
	ISDR≥2	10/19	1.0	11/19	0.59	10/19	1.0	11/19	1.0
	ISDR≤1	24/44		21/44		22/44		27/44	
Core	Wild core (Arg^{70}/Leu^{91})	23/31	0.01	22/31	0.02	20/31	0.13	24/31	0.005
	Non-wild-core	13/32		13/32		14/32		13/32	
	Gln^{70}	6/19	0.03	5/19	0.01	6/19	0.06	6/19	0.004
	Non-Gln^{70}	28/44		27/44		26/44		32/44	

Note: Patients Nos. 108, 111, 129, 135 and 152 were excluded from this analysis because their core antigen titers at certain time points were missing.

IRRDR = Interferon/ribavirin resistance-determining region; ISDR = interferon sensitivity-determining region; Arg^{70} = arginine at position 70; Leu^{91} = leucine at position 91; Gln^{70} = glutamine at position 70.

[a] Period after initiation of IFN/RBV therapy.

[b] Criteria of significant reduction of HCV core antigen titers.

(ISDR≥2 and ISDR≤1) and reduction of HCV core antigen titers during the very early stages of PEG-IFN/RBV therapy. On the other hand, non-wild-core or Gln^{70} were significantly associated with slow reduction and/or persistence of HCV core antigen in the patients' sera (table 6).

Identification of Independent Predictive Factors for SVR by Uni- and Multivariate Logistic Regression Analyses

Finally, in order to identify significant independent predictive factors of PEG-IFN/RBV treatment outcome, first, all available data of baseline patients' parameters, on-treatment responses and NS5A and core polymorphisms were entered in a univariate logistic analysis. This analysis yielded 11 factors that were correlated or nearly correlated with the treatment outcome; IRRDR mutations categorized as IRRDR≥6 and IRRDR≤5, Ala^{2360} and non-Ala^{2360}, core protein polymorphism categorized as wild-core (Arg^{70}/Leu^{91}) and non-wild-core, Gln^{70} and non-Gln^{70}, RVR and non-RVR, EVR and non-EVR, ETR and non-ETR, HCV core antigen titers, age, platelets count and hemoglobin levels (table 7). Subsequently, these 11 factors were entered in multivariate logistic regression analysis. This analysis yielded IRRDR mutations ($p = 0.005$), EVR ($p = 0.0001$) and age ($p = 0.02$) as independent predictive factors of PEG-IFN/RBV treatment outcome (table 7).

Discussion

Both host and viral genetic polymorphisms influence the outcome of PEG-IFN/RBV therapy for HCV-infected patients [15]. It has recently been reported that host genetic polymorphisms near or within the IL28B gene on chromosome 19 show a significant impact on the treatment outcome for patients infected with HCV genotype 1 (HCV-1a and -1b) [16–18]. Also, HCV genetic polymorphisms have been known to contribute to differences in the treatment outcome, as demonstrated by the observations that SVR rates for patients infected with HCV genotypes 2 and 3 are higher than those for patients infected with HCV genotype 1 [15]. Moreover, viral genetic polymorphisms, especially in the NS5A (ISDR and IRRDR) and the core regions, among HCV isolates of a given genotype have been linked to the difference in SVR rates [6–9, 19, 20]. In the present study, we compared the impact of IRRDR, ISDR and core polymorphisms of HCV-1b isolates on the clinical outcome of PEG-IFN/RBV therapy. Our results suggest that the degree of IRRDR mutations is more dominant than that of ISDR mutations and core polymorphism for predicting the anti-HCV treatment outcome.

IRRDR corresponds to a region near the C-terminus of NS5A. The obtained result that the IRRDR polymorphism influences the clinical outcome of IFN-based anti-HCV therapy can be linked to a recent experimental observation by Tsai et al. [21]. They reported that an HCV

Table 7. Uni- and multivariate logistic regression analyses to identify independent predictive factors for success of PEG-IFN/RBV combination therapy

Univariate		Multivariate	
variable	p value	odds ratio (95% CI)	p value
IRRDR mutations (IRRDR≥6 vs. IRRDR≤5)	<0.0001	14.33 (2.24–91.65)	0.005
Ala^{2360}	0.01	1.75 (0.19–15.36)	0.62
Core polymorphism (wild-core vs. non-wild-core)	0.06	0.41 (0.05–3.28)	0.34
Gln^{70}	0.04		
RVR	<0.0001		
EVR	<0.0001	41.83 (6.12–285.68)	0.0001
ETR	<0.0001		
HCV core antigen, fmol/l	0.05		
Age	0.01	0.91 (0.84–0.99)	0.02
Platelets, $\times 10^4/mm^3$	0.07		
Hemoglobin, g/dl	0.006		

IRRDR = Interferon/ribavirin resistance-determining region; Ala^{2360} = alanine at position 2360; Gln^{70} = glutamine at position 70; RVR = rapid virological response; EVR = early virological response; ETR = end-of-treatment response.

subgenomic RNA replicon containing NS5A of HCV-1b exerted more profound inhibitory effects on IFN activity than the original HCV-2a replicon, and that domain swapping between NS5A sequences of HCV-1b and -2a in the V3 and/or a C-terminus region including IRRDR resulted in a transfer of their anti-IFN activity. Since the C-terminal region of NS5A is among the most variable sequences across the different genotypes and subtypes of HCV [22], the difference in IFN responsiveness among different strains of a given HCV subtype could also be attributable, at least partly, to the genetic polymorphism within this region. The molecular mechanism underling the possible involvement of IRRDR in IFN responsiveness of the virus is still unknown. The significant difference in IRRDR sequence pattern may suggest genetic flexibility of this region and, indeed, the C-terminal portion of NS5A was shown to tolerate sequence insertions and deletions [23, 24]. This means that the C-terminal portion of NS5A is not essential for virus replication in cultured cells. It does not exclude the possibility, however, that the same region plays an important role in modulating the interaction with various host systems, including IFN responsiveness. It is also possible that the genetic flexibility of this region, especially IRRDR, is accompanied by compensatory changes elsewhere in the viral genome and that these compensatory changes affect overall viral fitness and responses to IFN therapy [25].

While we observed significant correlation between the overall number of mutations in IRRDR and PEG-IFN/RBV responsiveness, we also found a particular aa mutation, Ala^{2360}, that was significantly associated with SVR (tables 3, 7; fig. 1). It is possible that Ala or Val at this position confers a certain advantage for interaction between NS5A and the other viral or host proteins, which might affect IFN-induced antiviral responses. This issue needs to be elucidated in further studies.

The ISDR polymorphism was the only virological factor examined that showed a significant correlation with RVR (table 4), with the result being consistent with a recent report by other investigators [26]. This significant correlation, however, disappeared as the treatment went on. In contrast, the IRRDR polymorphism did not correlate significantly with RVR, however, it was the dominant viral genetic factor that was correlated with SVR (tables 3, 7). Interestingly, the combination of IRRDR and ISDR polymorphisms (IRRDR≥6 *plus* ISDR≥2) was significantly correlated with RVR and SVR (p = 0.0001 and 0.01, respectively; data not shown). This suggests a possible integrated influence of IRRDR and ISDR polymorphisms, or NS5A as a whole, on the treatment outcome. Further study is needed to clarify the issue.

The core protein polymorphisms (wild-core vs. non-wild-core, and Gln^{70} and non-Gln^{70}) were significantly correlated with the on-treatment HCV clearance kinetics

(fig. 2c, d; tables 4, 6). However, this significant correlation became blurred thereafter and eventually no significant correlation was observed between wild-core (Arg^{70}/Leu^{91}) and the final treatment outcomes (table 3). On the other hand, Gln^{70} was significantly associated with null-response, and almost significantly with non-SVR. This result is consistent, at least partly, with previous reports, including a recent multicenter study in Japan, that identified Gln^{70} as a predictive factor for poor responses to PEG-IFN/RBV treatment [8, 9, 14].

Recently, it was reported that the C-terminal region of NS5A plays a critical role in regulating the early phase of HCV particle formation [27, 28]. Moreover, sequence alteration within this region affected the degree of interaction between NS5A and core protein, which in turn affected the efficiency of progeny virus production [29]. In the present study, we observed a significant correlation between the degree of IRRDR mutations (IRRDR≥6) and the core polymorphism (table 5). Therefore, it would be interesting to investigate the degree of interaction between NS5A with IRRDR of high or low degrees of sequence variation and the wild-type (Arg^{70}/Leu^{91}) or non-wild-type of core protein, and also the impact of these interactions on progeny virus production and IFN sensitivity of the virus.

The present study identified the IRRDR polymorphism as the only viral genetic factor that independently predicted PEG-IFN/RBV treatment outcome (table 7). On the other hand, HCV is likely to utilize an alternative mechanism(s) by which to escape IFN actions through its various structural and non-structural proteins [30]. Also, a different lineage(s) of HCV-1b strains that relies more on the alternative mechanism than on IRRDR may prevail in other regions of the world. It is possible, therefore, that the impact of the IRRDR polymorphism differs with different cohorts. Analysis in a large-scale multicenter study is needed to clarify this issue.

In conclusion, NS5A (IRRDR and ISDR) and core protein polymorphisms are useful viral markers for predicting the outcome of PEG-IFN/RBV therapy for chronic hepatitis C. In particular, IRRDR≥6 is a useful marker for prediction of SVR.

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ORIGINAL ARTICLE

17β-estradiol inhibits the production of infectious particles of hepatitis C virus

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ABSTRACT

Persistent infection with hepatitis C virus causes serious liver diseases, such as chronic hepatitis, hepatic cirrhosis and hepatocellular carcinoma. The male gender is one of the critical factors in progression of hepatic fibrosis due to chronic HCV infection; thus female hormones may play a role in delaying the progression of hepatic fibrosis. It has also been reported that women are more likely than men to clear HCV in the acute phase of infection. These observations lead the present authors to the question: do female hormones inhibit HCV infection? In this study using HCV J6/JFH1 and Huh-7.5 cells, the possible inhibitory effect(s) of female hormones such as 17β-estradiol (the most potent physiological estrogen) and progesterone on HCV RNA replication, HCV protein synthesis and production of HCV infectious particles (virions) were analyzed. It was found that E_2, but not P_4, significantly inhibited production of the HCV virion without inhibiting HCV RNA replication or HCV protein synthesis. E_2–mediated inhibition of HCV virion production was abolished by a nuclear estrogen receptor (ER) antagonist ICI182780. Moreover, treatment with the ERα-selective agonist 4, 4′, 4″- (4-propyl-[1H]-pyrazole-1, 3, 5-triyl)trisphenol (PPT), but not with the ERβ-selective agonist 2, 3-bis (4-hydroxyphenyl)-propionitrile (DPN) or the G protein-coupled receptor 30 (GPR30)-selective agonist 1-(4-[6-bromobenzo 1, 3 dioxol-5-yl]-3a, 4, 5, 9b-tetrahydro-3H-cyclopenta [c] quinolin-8-yl)-ethanone (G-1), significantly inhibited HCV virion production. Taken together, the present results suggest that the most potent physiological estrogen, E_2, inhibits the production of HCV infectious particles in an ERα–dependent manner.

Key words 17β-estradiol, estrogen receptor, hepatitis C virus, sex difference.

HCV, an enveloped RNA virus which belongs to the genus *Hepacivirus* within the family *Flaviviridae*, prevails in most parts of the world with an estimated number of about 170 million carriers; hence HCV infection is a major global health-care problem (1). Persistent infection with HCV causes serious liver diseases, such as chronic hepatitis, hepatic cirrhosis and hepatocellular carcinoma (2, 3). In the USA, the prevalence of anti-HCV antibodies is twice as high in men as in women (4). The male gender is thought to be one of the critical factors in progression of hepatic fibrosis in chronic HCV infection (5, 6). It has also been reported that progression of hepatic fibrosis is faster in postmenopausal than in premenopausal women, and that hormone replacement therapy with estrogen and

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List of Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DPN, 2, 3-bis (4-hydroxyphenyl)-propionitrile; E_2, 17β-estradiol; ER, estrogen receptor; G-1, 1-(4-[6-bromobenzo 1, 3 dioxol-5-yl]-3a, 4, 5, 9b-tetrahydro-3H-cyclopenta [c] quinolin-8-yl)-ethanone; GPR30, G protein-coupled receptor 30; HCV, hepatitis C virus; P_4, progesterone; PPT, 4, 4′, 4″- (4-propyl-[1H]-pyrazole-1, 3, 5-triyl)trisphenol; SEM, standard error of the mean.

progesterone significantly delays progression of hepatic fibrosis in postmenopausal women (6, 7). This potential innate resistance of premenopausal women to hepatic fibrosis may be attributed to female hormones, such as estrogens and progesterone. In fact, E_2, the most potent physiological estrogen, has been reported to suppress the progression of liver fibrosis and hepatocarcinogenesis (8, 9). Moreover, women are more likely than men to clear HCV in the acute phase of infection, even within a few months after infection (10). These observations imply the possibility that female hormones inhibit HCV infection, either at the level(s) of virus attachment/entry, virus RNA replication, virus protein synthesis or production of infectious virus particles (virions).

Estrogens utilize three kinds of ER; ERα, ERβ and GPR30 (11–15). Specific agonists and antagonists of ER are available and widely used to examine the roles of estrogens. In the present study, we examined the possible effects of female hormones, especially E_2 and P_4, on HCV RNA replication, protein synthesis and virion production in cultured cells.

MATERIALS AND METHODS

Cell culture and virus infection

A human hepatoma-derived cell line, Huh-7.5, which is highly permissive to HCV RNA replication (16), was kindly provided by Dr. C. M. Rice (The Rockefeller University, New York, NY, USA). The cells were maintained in phenol red-free DMEM (Sigma–Aldrich, St Louis, MO, USA) supplemented with 10% heat-inactivated and charcoal-stripped FBS (Israel Beit Haemek, Haemek, Israel), 0.1 mM non-essential amino acids (Invitrogen, Carlsbad, CA, USA), 100 IU/mL penicillin and 100 μg/mL streptomycin (Invitrogen).

The pFL-J6/JFH1 plasmid that encodes the entire viral genome of a chimeric strain of HCV-2a, J6/JFH1 (17) was kindly provided by Dr. C. M. Rice. A cell culture-adapted mutant derived from J6/JFH1 (P-47 strain) (18, 19) was used for infection experiments. The virus was inoculated into Huh-7.5 cells at a multiplicity of infection of 1.0 and incubated for 2 hr. After the residual virus had been removed by washing, the cells were cultured in the presence or absence of female hormones, and agonists and an antagonist of estrogen receptors (see below). Culture supernatants were collected at 0, 1, 2 and 3 days postinfection and virus titers were determined, as described below.

Virus titration

Culture supernatants containing HCV were serially diluted 10-fold in DMEM and inoculated into Huh-7.5 cells (2×10^5 cells per well in a 24-well plate). After incubation at 37°C for 6 hr, the cells were fed with fresh DMEM. At 24 hr postinfection, the cells were fixed with ice-cold methanol, blocked with 5% goat serum in PBS and subjected to immunofluorescence analysis using mouse monoclonal antibody against the HCV core protein (2H9) and Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L, Molecular Probes, Eugene, OR, USA). Hoechst 33342 (Molecular Probes) was used for counterstaining of the nuclei. HCV-positive foci were counted under a fluorescent microscope (BX51; Olympus, Tokyo, Japan) and virus titers were expressed as focus-forming units per ml, as reported previously (18, 19).

Chemicals

E_2 and P_4 were purchased from Sigma–Aldrich (St Louis, MO, USA). ICI182780 (an antagonist of ERα and ERβ), PPT (an ERα-selective agonist) (20) and DPN (an ERβ-selective agonist) (21) were purchased from Tocris Bioscience (Bristol, UK). G-1 (a GPR30-selective agonist) (22) was purchased from Calbiochem (Darmstadt, Germany). DMSO, which was used as a solvent, was obtained from Wako Pure Chemical Industries (Osaka, Japan). The concentrations of E_2 and P_4 used in this study were 0.4 μM and 3 μM, respectively, which correspond to the estimated highest concentrations in the sera of pregnant women. ICI182780 was used at a concentration of 1 μM, PPT and DPN at 0.1, 1 and 10 μM, and G-1 at 0.1 and 1 μM. As G-1 has been reported to lose its GPR30-binding specificity at concentrations over 1 μM, a concentration of 10 μM for G-1 was not tested. The final concentration of DMSO as a control never exceeded 0.01%.

Cell viability assay

Cells plated on 96-well microtiter plates (2.0×10^4 cells/well) were inoculated with HCV and treated with E_2, P_4 or DMSO. The cell viability in each well was determined by WST-1 assay (Roche Diagnostics, Mannheim, Germany) until 3 days postinfection.

Real-time quantitative RT-PCR

Total cellular RNA was isolated using the RNAiso reagent (Takara Bio, Kyoto, Japan) and cDNA was generated using the QuantiTect Reverse Transcription system (Qiagen, Valencia, CA, USA). Real-time quantitative PCR was performed on a SYBR *Premix Ex Taq* (Takara Bio) using SYBR green chemistry in ABI PRISM 7000 (Applied Biosystems, Foster, CA, USA). Primer sets used in this study are shown below: HCV NS5B, 5′-ACCAAGCTCAAACTCACTCCA-3′ and 5′-AGCGGGGTCGGGCAC GAGACA-3′ (23);

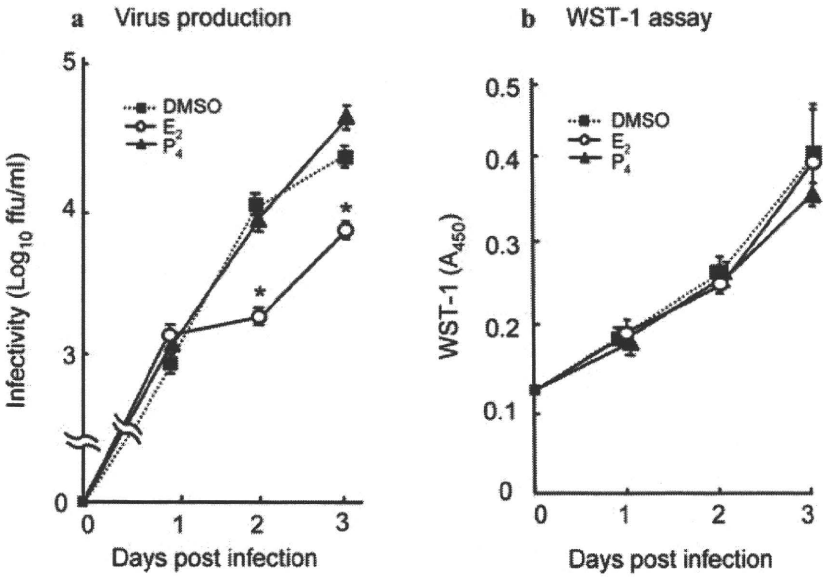

Fig. 1. Effects of E_2 and P_4 on HCV virion production and cell growth. (a) HCV virion production. Huh-7.5 cells were inoculated with HCV at a multiplicity of infection of 1.0, incubated for 2 hr, and cultured for 0, 1, 2 and 3 days after virus infection. The HCV-infected cells were treated with E_2 (0.4 μM), P_4 (3 μM) or DMSO (control) from 2 hr postinfection to sampling time (days 1, 2 and 3). The culture supernatants of HCV-infected cells were assayed for virus infectivity. Data are shown as mean ± SEM. (b) Cell growth. HCV-infected cells were treated with E_2, P_4 or DMSO (control) from 2 hr to 3 days postinfection. Cell growth in each culture was determined by WST-1 assay. Data are shown as mean ± SEM.

β-actin, 5′-GCGGGAAATCGTGCGTGACATT-3′ and 5′-GATGGAGTTGAAGGTAGTTTCGTG-3′.

Immunoblotting

Cells were solubilized in lysis buffer as reported previously (18, 19). The cell lysates were subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membranes were incubated with mouse monoclonal antibodies against HCV NS3 (Chemicon International, Temecula, CA, USA), followed by incubation with peroxidase-conjugated goat anti-mouse IgG (Medical & Biological Laboratories Co. Ltd., Nagoya, Japan). The positive bands were visualized by using ECL detection system (GE Healthcare UK, Buckinghamshire, UK).

Statistical analysis

Results were expressed as mean ± SEM. Statistical significance was evaluated by one-way analyses of variances.

RESULTS

E_2 inhibits HCV virion production, but not HCV RNA replication or HCV protein synthesis

We first examined the effect of E_2 or P_4 treatment on HCV virion production. At 2 hr after virus inoculation, the HCV-infected Huh-7.5 cells were treated with E_2 (0.4 μM) or P_4 (3 μM) for 3 days. Culture supernatants were collected every day and titrated for viral infectivity. As shown in Figure 1a, E_2 treatment significantly suppressed HCV virion production at 2 and 3 days postinfection, whereas treatment with P_4 did not. The same treatment (E_2 or P_4) did not exert significant cytotoxicity (Fig. 1b). Next, we examined the effect of E_2 on HCV RNA replication and HCV protein synthesis under the same experimental conditions. We found that HCV RNA replication and HCV protein synthesis in both HCV-infected cells and HCV RNA replicon-harboring cells (23) were all unaffected by treatment with E_2 or P_4 (Fig. 2a–c). Moreover, treatment of the cells with E2 either prior to, or during, virus inoculation did not significantly inhibit HCV virion production (Fig. 3a). These results collectively suggest that E_2 inhibits HCV virion production, but not at the level of virus entry, RNA replication or protein synthesis. We also observed that E_2 –mediated inhibition of HCV virion production occurs in a dose-dependent manner (Fig. 3b).

A nuclear estrogen receptor antagonist, ICI182780, abolishes E_2-mediated inhibition of HCV virion production

We hypothesized that E_2 signaling through nuclear ER (ERα and ERβ) was involved in the E_2–mediated inhibition of HCV virion production. To test this possibility, we used ICI182780 (1 μM), an antagonist of ERα and ERβ. The results clearly demonstrated that treatment of cells with ICI182780 abolished E_2–mediated inhibition of HCV virion production (Fig. 4).

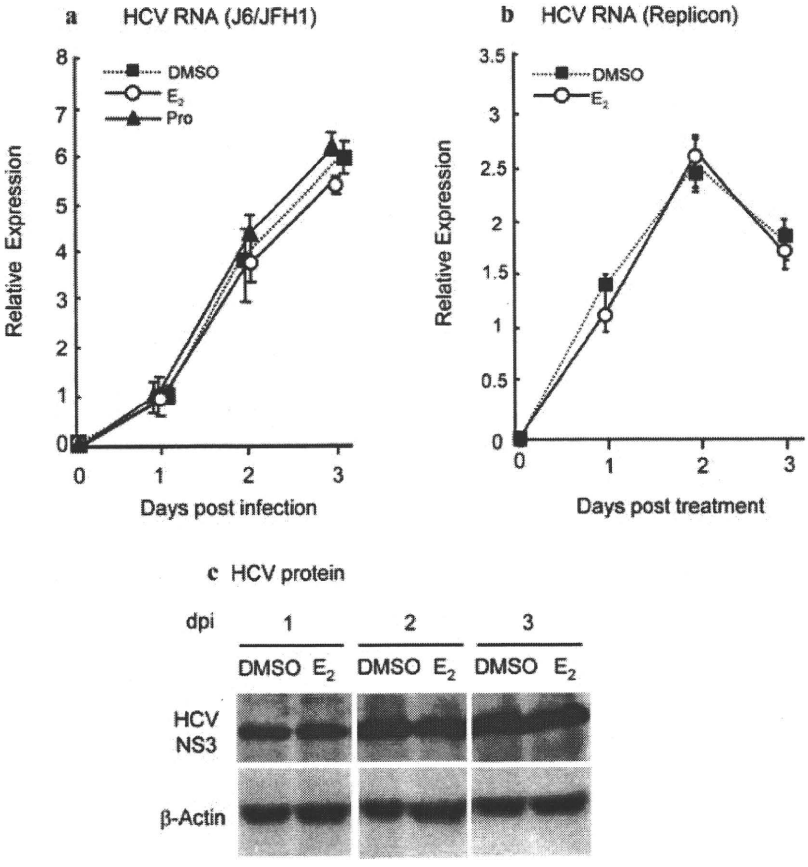

Fig. 2. Effects of E_2 and P_4 on HCV RNA replication and HCV protein synthesis. (a) HCV RNA replication. Huh-7.5 cells were inoculated with HCV at a multiplicity of infection of 1.0, incubated for 2 hr, and cultured for 0, 1, 2 and 3 days after virus infection. The HCV-infected cells were treated with E_2 (0.4 μM) or DMSO (control) from 2 hr to sampling time (days 1, 2 and 3). HCV RNA replication levels were determined by real-time quantitative RT-PCR and normalized with β-actin mRNA levels. Data are shown as mean ± SEM. (b) Huh-7.5 cells harboring a full-genomic HCV RNA replicon (23) were treated with E_2 (0.4 μM) or DMSO, and HCV RNA replication levels determined as in (a). (c) HCV protein synthesis. HCV-infected cells were treated with E2 or DMSO as in (a) and the amount of HCV protein synthesis determined by immunoblot analysis using anti-NS3 antibody. The degree of β-actin expression as determined by anti-β-actin antibody served as a control. dpi, days postinfection.

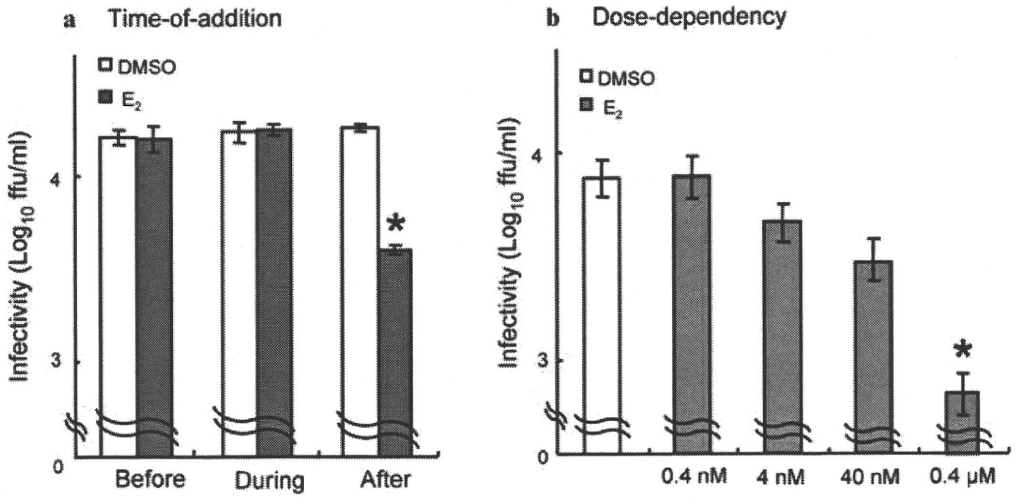

Fig. 3. Kinetic analysis of E_2–mediated inhibition of HCV virion production. (a) Time-of-addition experiment. Huh-7.5 cells were inoculated with HCV at a multiplicity of infection of 1.0, incubated for 2 hr, and cultured up to 2 days after virus infection. Treatment of the cells with E_2 (0.4 μM) was performed before or during virus inoculation for 2 hr, or after virus inoculation until sampling time (day 2). The culture supernatants of HCV-infected cells were assayed for viral infectivity. Data are shown as mean ± SEM. *$P < 0.05$, compared with DMSO control. (b) Dose–dependency experiment. Huh-7.5 cells were inoculated with HCV as in (a). The HCV-infected cells were treated with various concentrations of E_2 (0.4 nM to 0.4 μM]) from 2 hr postinfection to sampling time (day 2). The culture supernatants of HCV-infected cells were assayed for viral infectivity. Data are shown as mean ± SEM. *$P < 0.05$, compared with DMSO control.

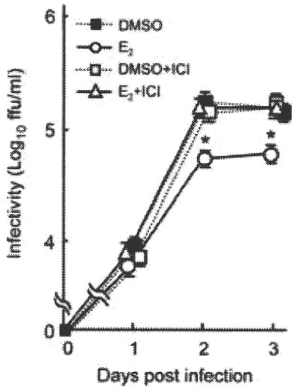

Fig. 4. Effects of ER antagonist, ICI182780, on HCV virion production. Huh-7.5 cells were inoculated with HCV at a multiplicity of infection of 1.0, incubated for 2 hr, and cultured for 0, 1, 2 and 3 days after virus infection. The HCV-infected cells were treated with E_2 (0.4 μM) and/or ICI182780 (1 μM) or DMSO (control) from 2 hr postinfection to sampling time (days 1, 2 and 3). The culture supernatants of HCV-infected cells were assayed for virus infectivity. Data are shown as mean ± SEM. *$P < 0.05$, compared with DMSO control.

Estrogen receptor-α-selective agonist 4, 4′, 4″- (4-propyl-[1H]-pyrazole-1, 3, 5-triyl) trisphenol inhibits HCV virion production

To determine which estrogen receptor(s) is/are involved in the E_2–mediated down-regulation of HCV virion production, we used receptor-specific agonists, such as PPT (an ERα-selective agonist) (20), DPN (an ERβ-selective agonist) (21) and G-1 (a GPR30-selective agonist) (22). Treatment of cells with PPT (10 μM), but not with DPN (10 μM) or G-1 (1 μM), significantly inhibited HCV virion production (Fig. 5). PPT treatment at a concentration of 1 μM also brought about a weak, but significant, inhibition of HCV virion production at 2 days postinfection. On the other hand, PPT did not mediate significant cytotoxicity at the concentrations tested (data not shown).

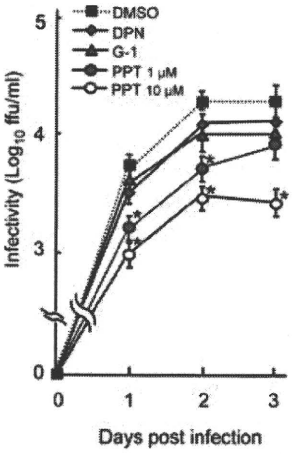

Fig. 5. Effects of ER-specific agonists on HCV virion production. Huh-7.5 cells were inoculated with HCV at a multiplicity of infection of 1.0, incubated for 2 hr, and cultured for 0, 1, 2 and 3 days after virus infection. The HCV-infected cells were treated with PPT (ERα-selective agonist; 1 and 10 μM), DPN (ERβ-selective agonist; 10 μM) or G-1 (GPR30-selective agonist; 1 μM) from 2 hr postinfection to sampling time (days 1, 2 and 3). The culture supernatants of HCV-infected cells were assayed for viral infectivity. Data are shown as mean ± SEM. *$P < 0.05$, compared with DMSO control.

DISCUSSION

We have demonstrated in the present study that treatment of Huh-7.5 cells with E_2 inhibits HCV virion production, but not HCV RNA replication or HCV protein synthesis (Figs 1 and 2). Treatment of the cells with E_2 either prior to, or during, virus inoculation did not significantly suppress HCV virion production (Fig. 3a). These results collectively suggest that E_2 inhibits HCV infection at the virion assembly/secretion level, but not at the level of virus attachment/entry, virus RNA replication or virus protein synthesis. E_2 has been reported to possess antioxidant and anti-apoptotic activities in fibrotic liver and cultured hepatocytes (24, 25). It should be noted, however, that E_2 did not exert anti-apoptotic or cytotoxic (pro-apoptotic) effect under our experimental conditions (Fig. 1b). In contrast to E_2, another female hormone, P_4, did not significantly affect HCV virion production (Fig. 1a).

E_2-mediated inhibition of HCV virion production was abolished by a nuclear ER (ERα and ERβ) antagonist, ICI182780 (Fig. 4), this result suggesting that suppression of HCV virion production may be induced by ER signal transduction. Three types of ER have been reported so far; ERα, ERβ and GPR30 (11–15). To determine which ER is involved in the suppression of HCV virion production, we used ER-specific agonists, PPT (for ERα) (20), DPN (for ERβ) (21) and G-1 (for GPR30) (22). We found that PPT, but not DPN or G-1, inhibits the production of HCV infectious particles (Fig. 5), suggesting that ERα plays an important role in the inhibition of HCV virion production. It has been reported that, in hepatocytes, ERα constitutes a minor proportion of the total ER, and that an estrogen-mediated anti-apoptotic effect is mediated principally through ERβ (26). However, the importance of ERα–mediated signal transduction should not be ignored. The rationale for this assertion is that ERα is known to be involved in lipid metabolism (27), that certain lipid metabolism disorder(s) possibly result(s) in abnormal accumulation of lipid droplets, and that such an accumulation is required for HCV virion maturation in virus-infected cells (27), that certain lipid metabolism disorder(s) possibly result(s) in abnormal accumulation of

lipid droplets, and that such an accumulation is required for HCV virion maturation in virus-infected cells (28). Also, we should not yet exclude the possible importance of ERβ and GPR30, because they may not be expressed at a sufficient level in the Huh7.5 cell line maintained in our laboratory.

Other relevant observations are that ERα interacts with HCV NS5B, the viral RNA polymerase, and promotes association of NS5B with the replication complex in human hepatoma-derived Huh-7 cells, and that tamoxifen, a competitive inhibitor of estrogens, suppresses the ERα–mediated association of NS5B with the replication complex, thereby inhibiting HCV RNA replication (29). Similarly, E_2 binding to ERα may abrogate its interaction with NS5B. However, in our experiments we did not observe E_2–mediated inhibition of HCV RNA replication (Fig. 2a,b). We therefore assume that E_2 inhibits HCV virion production through a mechanism other than E_2–ERα–NS5B interactions. Further study is needed to elucidate this issue.

In conclusion, the most potent physiological estrogen, E_2, inhibits production of HCV infectious particles in Huh-7.5 cell cultures in an ERα–dependent manner. This may explain, at least in part, why the incidence of HCV-associated liver disease is lower in premenopausal women than in postmenopausal women and men.

ACKNOWLEDGMENTS

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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.

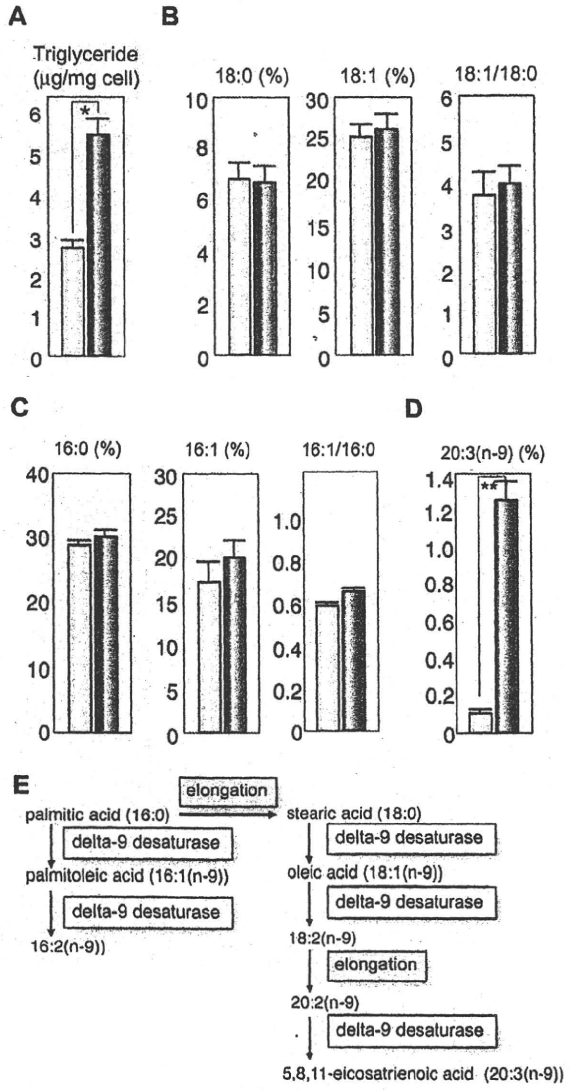

Fig. 1. Effect of the core protein on fatty acid composition in HepG2 cells. The fatty acid compositions of the total cell lipids were analyzed and the ratios of 18:1/18:0 and 16:1/16:0 in the core-expressing and control HepG2 cells were calculated. (A) Concentrations of triglycerides. (B) Percentages 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 (Hep39], 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.

Research Article

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

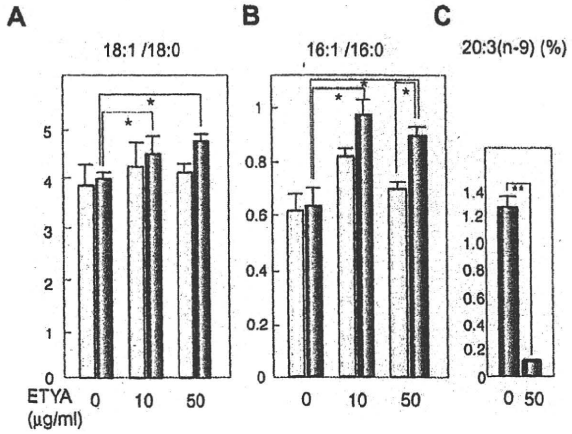

Fig. 2. Effect of ETYA on delta-9 desaturase index. HepG2 cells with or without the core protein were incubated with ETYA for 48 h. The fatty acid compositions of the total cell lipids were analyzed, and the ratios of 18:1/18:0 (A) and 16:1/16:0 (B), and the percentage of eicosatrienoic acid (20:3(n-9)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

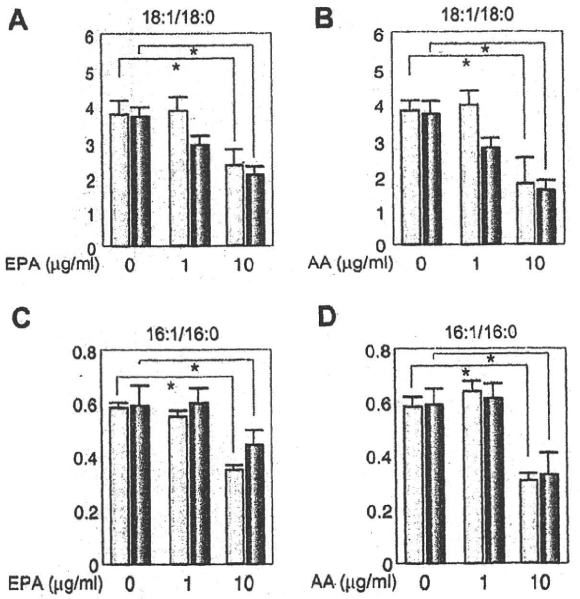

Fig. 3. Effect of EPA and AA on delta-9 desaturase index. HepG2 cells with or without the core protein were incubated with EPA (A and C) or AA (B and D) for 48 h. The fatty acid compositions of the total cell lipids were analyzed and the ratios of 18:1/18:0 (A and B) and 16:1/16:0 (C and D) were computed.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

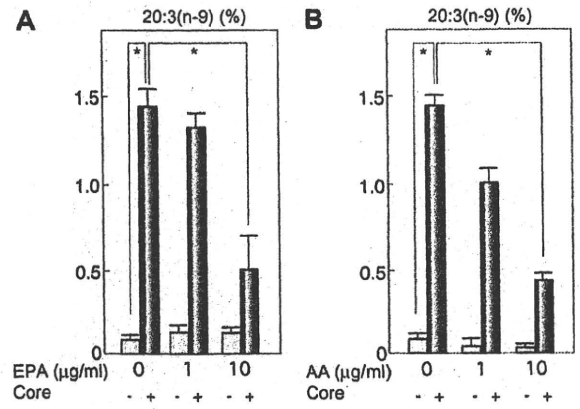

Fig. 4. Effect of EPA and AA on the concentration of 20:3(n-9). HepG2 cells with or without the core protein were incubated with EPA (A) or AA (B) for 48 h. The fatty acid compositions of the total cell lipids were analyzed and the percentages of the C20:3(n-9) fraction were measured. Light blue bars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

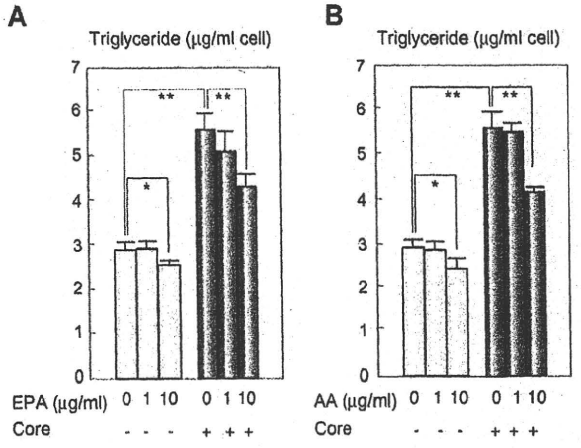

Fig. 5. Effect of EPA and AA on triglyceride content. HepG2 cells with or without the core protein were incubated with EPA (A) or AA (B) for 48 h. The triglyceride volume of the total cell lipids was measured and the triglyceride contents in the cells were calculated. Light blue bars indicate control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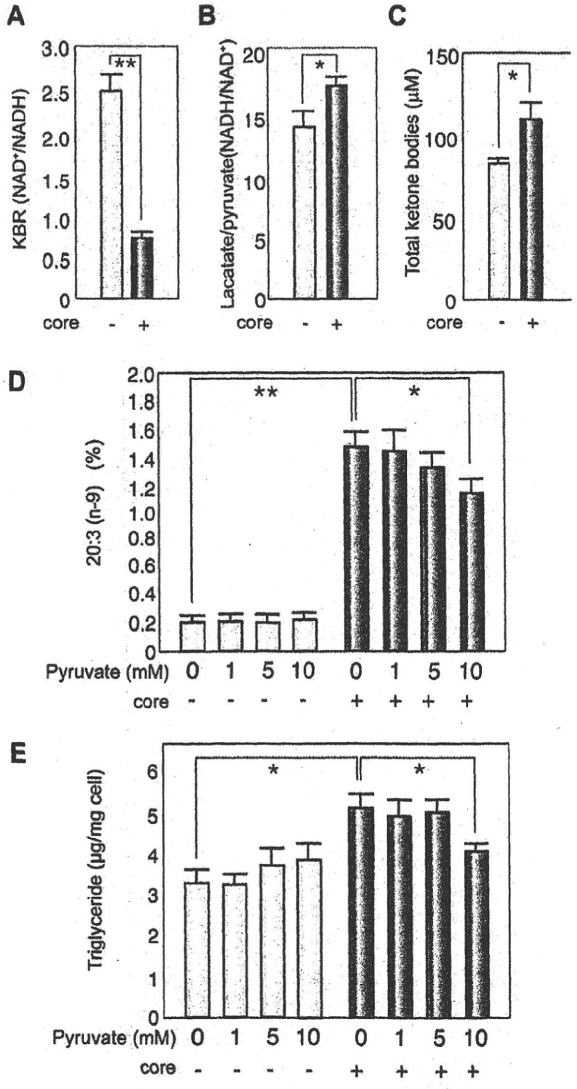

Fig. 6. NADH accumulation and effect of pyruvate in core-expressing cells. HepG2 cells with or without the core protein were subjected to the determination of ketone body ratio (A) and lactate/pyruvate ratio (B) for the precise estimation of $NAD^+/NADH$ and $NADH/NAD^+$. (C) Total ketone bodies. (D)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

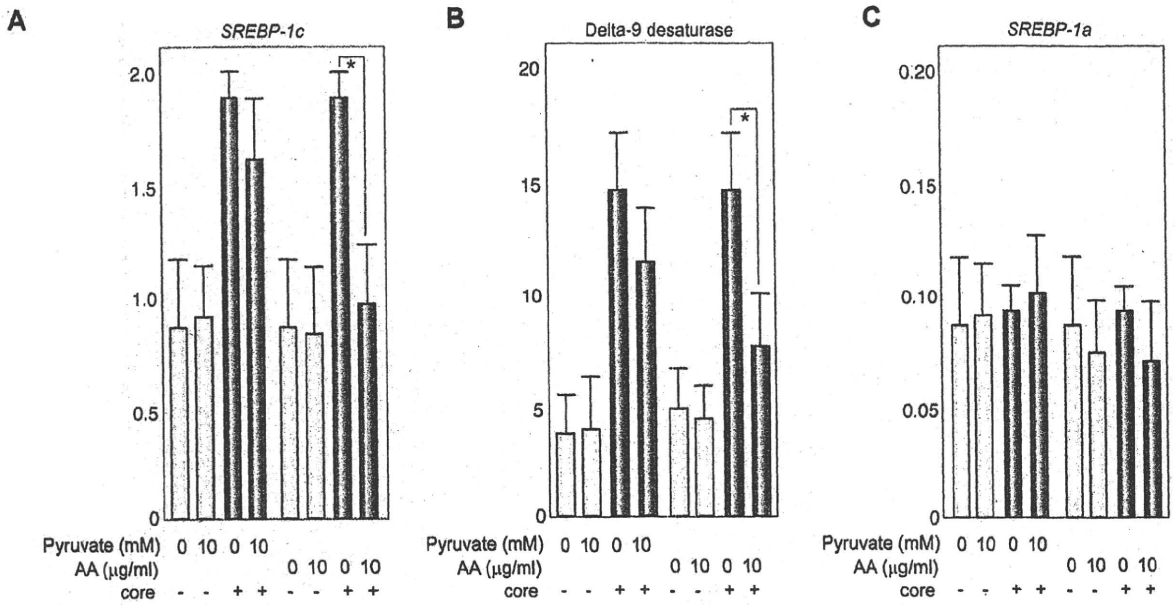

Fig. 7. Effect of pyruvate and AA on mRNA levels of lipid-associated genes. The mRNA levels of *SREBP-1c* (A), delta-9 desaturase (B) and *SREBP-1a* (C) genes were determined by real-time PCR analysis. The transcription of the genes was normalized with that of hypoxanthine phosphoribosyltransferase,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

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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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