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HCV core protein induces hepatic lipid accumulation by activating SREBP1 and PPARγ

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Abstract

Hepatic steatosis is a common feature in patients with chronic hepatitis C virus (HCV) infection. HCV core protein plays an important role in the development of hepatic steatosis in HCV infection. Because SREBP1 (sterol regulatory element binding protein 1) and PPARγ (peroxisome proliferators-activated receptor γ) are involved in the regulation of lipid metabolism of hepatocyte, we sought to determine whether HCV core protein may impair the expression and activity of SREBP1 and PPARγ. In this study, it was demonstrated that HCV core protein increases the gene expression of SREBP1 not only in Chang liver, Huh7, and HepG2 cells transiently transfected with HCV core protein expression plasmid, but also in Chang liver-core stable cells. Furthermore, HCV core protein enhanced the transcriptional activity of SREBP1. In addition, HCV core protein elevated PPARγ transcriptional activity. However, HCV core protein had no effect on PPARγ gene expression. Finally, we showed that HCV core protein stimulates the genes expression of lipogenic enzyme and fatty acid uptake associated protein. Therefore, our finding provides a new insight into the mechanism of hepatic steatosis by HCV infection.

Keywords: HCV; Core protein; SREBP1; PPARγ; Hepatic steatosis

Chronic hepatitis C virus (HCV) infection is a major cause of chronic liver disease worldwide, affecting about 3% of the population. The HCV infection is characterized by a high rate of progression to fibrosis, chronic hepatitis, leading to cirrhosis and ultimately to hepatocellular carcinoma [1]. In addition, various observations suggest that hepatic steatosis is a common histologic feature of chronic infection with HCV [2]. Furthermore, increasing evidence indicates that hepatic steatosis is a more vulnerable factor that leads to liver inflammation and fibrosis [3]. These suggest that HCV has a direct role in the development of steatosis and/or that the presence of steatosis affects the progression of HCV-related liver disease. The core protein component of HCV is known to contribute to hepatic steatosis [4,5], hepatic fibrosis [6], and hepatic carcinogenesis [7]. Some studies suggest that HCV core protein causes hepatic steatosis through inhibition of microsomal triglyceride transfer protein (MTP) activity and very low density lipoprotein (VLDL) secretion [8], and impairment of the expression and transcriptional activity of PPARα [9].

Sterol regulatory element binding protein 1s (SREBP1s) belong to the basic helix-loop-helix-leucine zipper family of transcription factors [10]. SREBP1s are synthesized as precursors (~125 kDa) bound to the endoplasmic reticulum and nuclear envelope. Upon activation, SREBP1s are released from the membrane into the nucleus as a mature protein (~68 kDa) by a sequential two-step cleavage process. To date, two SREBP1 isoforms (SREBP1a and SREBPlc) have been identified and characterized. A key role of two SREBP1 isoforms in regulating fatty acid synthesis in liver is suggested by study of transgenic mice

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overexpressing the constitutively active mature forms of SREBP1 isoforms [11]. These transgenic mice study suggests that two SREBP1s increase the transcription of genes involved in hepatic fatty acid synthesis (including FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase; SCD, stearoyl-CoA desaturase), inducing massive hepatic steatosis through increased accumulation of triglycerides.

Peroxisome proliferator-activated receptor (PPAR) γ is a transcription factor, belonging to the nuclear receptor superfamily, and is a master regulator for adipocyte differentiation, and is important in regulation of a number of genes involved in fatty acid and glucose metabolism [12]. Similar to SREBP1, PPARγ mediates the development of hepatic steatosis in the mouse models [13,14]. There evidences suggest that liver PPARγ increases the transcription of genes involved in hepatic fatty acid synthesis (including FAS, ACC, SCD) and fatty acid uptake (including FAT/CD36, fatty acid translocase). Thus, liver PPARγ contributes to regulation of lipid synthesis, transport, and storage within the hepatocytes, causing the development of hepatic steatosis.

In view of the key role of HCV core protein in hepatic steatosis and the importance of SREBP1 and PPARγ in lipid metabolism, we investigated whether expression and activity of SREBP1 and PPARγ may be deregulated, and therefore, might play a role in HCV core-induced hepatic triglycerides deposit. We showed that HCV core protein increases hepatic lipid accumulation via activation of SREBP1 and PPARγ. Our results suggest that the deregulation of SREBP1 and PPARγ may be influenced by HCV core protein and may be involved in HCV core-induced hepatic steatosis.

Materials and methods

Plasmid constructs and reagents. pCAG-GS-HA-core173 and pCAG-GS-HA-core191 (HCV genotype1b) were kindly gifted from Dr. Matsuura. Y. pcDNA3.1-Flag-SREBP1a (human, aa 1–490), pGL2B-FAS-luc (−250 to +65) and pSynSRE-luc were kindly gifted from Dr. T. F. Osborne. A human mature SREBP1c cDNA (aa 1–447) fragment was cloned by reverse transcription-PCR using HepG2 mRNA and a pair of primers: Forward 5′-gcggaattcggagccatggattgcactttcga-3′, Reverse 5′-gcctcgagctatggctttgccttgctgtcctc-3′. The PCR products were digested with *Eco*RI and *Xho*I and were inserted into *Eco*RI and *Xho*I digested pcDNA3-HA. pcDNA3-HA-core191 and pcDNA3-HA-core173 were constructed by inserting PCR fragments of ORF into *Eco*RI and *Xho*I digested pcDNA3-HA. PPARγ2 expression plasmid and PPRE reporter plasmid were described previously [15]. All plasmids were confirmed by automatic sequencing analysis. G418 was purchased from A.G. Scientific. The transfection reagent SuperFect and Fugene6 were purchased from Qiagen and Roche, respectively. All other reagents were purchased from Sigma.

Cell culture. Chang liver (ATCC CCL 13), HepG2, and Huh7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C in a humid atmosphere of 5% CO_2. Chang-core191 stable cells were maintained in DMEM supplemented with 10% FBS containing antibiotics G418.

Establishment of Chang liver cells expressing core191 protein. Chang liver cells were seeded in 6-well culture plates for 24 h prior to transfection. Cells were transfected with 2 μg of pcDNA3-HA or pcDNA3-HA-core191 using the SuperFect according to the manufacturer's instructions. After 48 h, cells were trypsinized and plated in a medium containing 500 μg/ml G418. Following selection for 2 weeks, total populations of G418-resistant cells were pooled and single-cell sorted into 96-well plates with a growth medium containing 500 μg/ml G418. Sorted single cells were grown under selection for an additional 2 weeks and expanded into stable cell lines. The candidate clones were analyzed by western blotting using specific HA-antibody (Roche). The selected permanent cell clones were maintained in the presence of G418 (500 μg/ml) throughout the experiment.

Western blotting and antibodies. Cells were lysed in a lysis buffer containing 150 mM NaCl, 50 mM Tris–Cl, pH 7.5, 1 mM EDTA, 1% Nonidet P-40, 10% glycerol and protease inhibitors for 20 min on ice. The protein concentration was determined by the Bradford assay (Bio-Rad). Fifty micrograms of protein from whole cell lysates was subjected to 10% SDS–PAGE and transferred to a PVDF membrane (Millpore) by semidry electroblotting. The membranes were then incubated for 2 h at room temperature with anti-SREBP1 antibody (sc-13551, Santa Cruz), anti-TBP antibody (sc-273, Santa Cruz), or anti-HA antibody (Roche) in TBST supplemented with 1% nonfat dry milk. The bands were detected using an enhanced chemiluminescence system (Amersham).

Luciferase reporter assay. Cells were seeded in a 24-well culture plate and transfected with reporter vector (0.2 μg) and β-galactosidase expression plasmid (0.2 μg), along with each indicated expression plasmids. The pcDNA3.1/HisC plasmid was added to the transfections, as needed, to achieve the same total amount of plasmid DNA per transfection. After 48 h of transfection, the cells were lysed in the cell culture lysis buffer (Promega). Luciferase activity was determined using an analytical luminescence luminometer according to the manufacturer's instructions. Luciferase activity was normalized for transfection efficiency using the corresponding β-galactosidase activity. Data shown are means ± SD of three independent experiments performed in duplicate.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from various transfected Chang liver cells was prepared using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was converted into single strand cDNA by AMV reverse transcriptase (Takara, Japan) with an oligo(dT) adaptor primer. A one-fourth aliquot of the cDNA was subjected to PCR amplification using gene-specific primers: FAS, 5′-gaaactgcaggagctgtc-3′ (forward) and 5′-cacggagttgaggcggat-3′ (reverse); ACC, 5′-gctgctcggatcactag tgaa-3′ (forward) and 5′-ttctgctatcagtctgtccag-3′ (reverse); SCD, 5′-cctcta cttggaagacgacattcgc-3′ (forward) and 5′-gcagccgagctttgtaagagcggt-3′ (reverse); SREBP1, 5′-acggcagcccctgtaacgaccactgtga-3′ (forward) and 5′-tgccaagatggttccgccactcaccagg-3′ (reverse); PPARγ, 5′-gaaatgaccatggttgac-3′ (forward) and 5′-ccgctagtacaagtccttgta-3′ (reverse); CD36, 5′-acgct gaggacaacacagtctct-3′ (forward) and 5′-gtaacagggtacggaaccaaactc-3′ (reverse); HCV core, 5′-*cggaattc*atgagcacaaatcctaaaccc-3′ (forward) and 5′-*gactcgag*agcggaagctgggatggtcaa-3′ (reverse); β-actin, 5′-gactacctcatga agatc-3′ (forward) and 5′-gatccacatctgctggaa-3′ (reverse). The RT-PCR bands were quantified and normalized relative to the β-actin mRNA control band with ImageJ version 1.35d (NIH Image).

Oil-Red O staining. Cells were stained with Oil Red O using standard procedures. Briefly, cells were fixed in 10% solution of formaldehyde in aqueous phosphate buffer for 1 h or more, washed with 60% isopropanol, and stained with Oil Red O solution (in 60% isopropanol) for 10 min followed by repeated washing with distilled water. The cells were then photographed using microscopy.

Results

HCV core protein increases the gene expression of SREBP1

To better study the role of HCV core protein in hepatic lipid accumulation, we established Chang liver cells having stable expression of HCV core191 protein. The expression of HCV core mRNA and protein were confirmed by RT-PCR and immunoblotting, respectively

(data not shown). To examine the effect of HCV core protein on the lipid accumulation, Chang-core stable cells and parent Chang live cells were stained with Oil-Red O (ORO). The percentage of ORO-positive cells among Chang liver cells stably expressing HCV core protein was significantly higher than in parent Chang liver cells (data not shown). Because SREBP1 has been shown to play a critical role for hepatic lipid accumulation, we investigated the effect of HCV core protein on the gene expression of SREBP1. We observed that HCV core protein increases the expression of SREBP1 mRNA or premature/mature SREBP1 (pSREBP1/mSREBP1) protein in Chang-core191 stable cells (Fig. 1A) and transiently transfected Chang liver cells (Fig. 1B and C). It is reported that mature HCV core protein (C-terminal truncated, 173 aa) is the major form present in the native viral particles together full length core protein (191 aa) [16]. We examined whether mature core protein also modulates the gene expression of SREBP1. As shown in Fig. 1B and C, the truncated core protein also induced the transcription of SREBP1 gene in Chang liver cells. This enhancement by the core protein was also observed in HepG2 and Huh7 (data not shown). Thus, these results indicate that HCV core protein elevates the mRNA and protein expression of SREBP1.

HCV core protein enhances the transcriptional activity of SREBP1

The potential capacity of SREBP1 as a transcriptional regulator is modulated not only in protein level but also in transcriptional activity. We next examined whether HCV core protein would also affect the transcriptional activity of SREBP1. Since fatty acid synthase (FAS) was identified as a novel target gene of SREBP1, FAS-promoter reporter construct was co-transfected in Chang liver and Huh7 cells together with HCV core protein expression vector and SREBP1a/1c expression plasmids. As shown in Fig. 2A, the full-length core protein significantly increased the luciferase activity of FAS promoter by exogenous SREBP1a/1c in Chang liver and Huh7 cells. Furthermore, the mature core protein also enhanced the transcriptional activity of exogenous SREBP1a/1c in two cell lines (data not shown). In addition, to test whether HCV core protein regulates the activity of endogenous SREBP1, we utilized the pSyn-SRE luciferase plasmid containing SREBP-response elements (SRE). As shown in Fig. 2B, the two core protein forms reinforced SRE-dependent luciferase activity. Taken together, these data suggest that HCV core protein plays a critical role as a regulator enhancing the transcriptional activity of SREBP1.

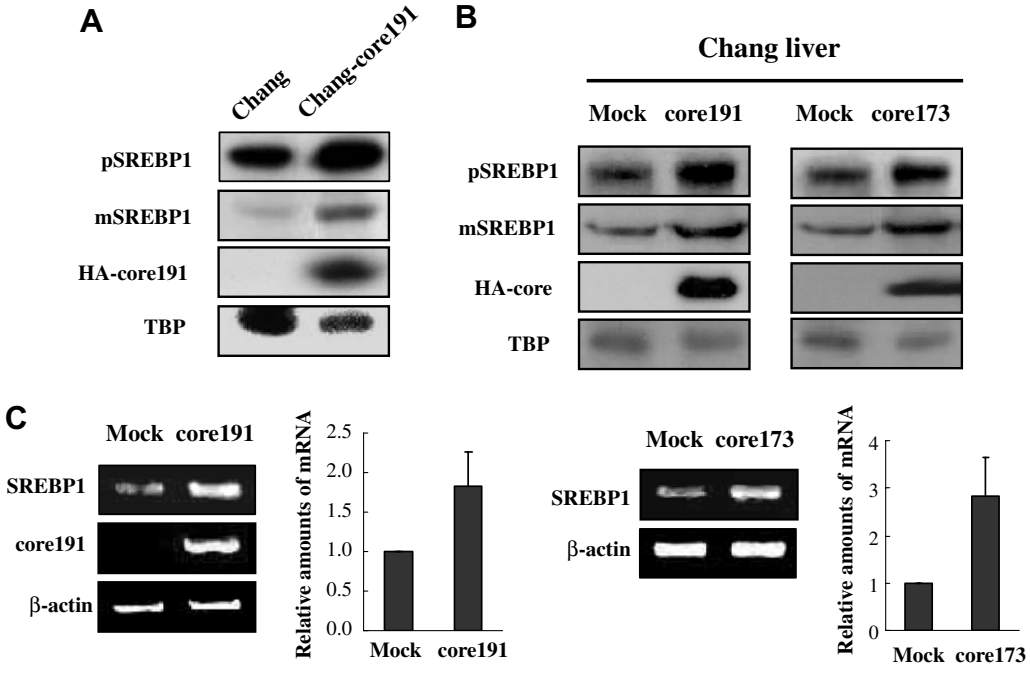

Fig. 1. The effect of HCV core protein on the expression of SREBP1 gene. (A) The protein expression of SREBP1 in Chang liver core191 stable cells. Cell lysates were prepared and analyzed for the expression of HA-core191, SREBP1 and TBP using specific antibodies by immunoblotting as described. (B,C) The increase of SREBP1 gene expression in Chang liver cells transiently transfected with core191 and core173 protein expression vectors. Cells were transfected with indicated expression plasmids. After 48 h of transfection, cell lysates were analyzed for expression of the indicated proteins by immunoblotting. Data shown are representative of three independent experiments. To analyze transcripts, cells were transfected with indicated expression plasmids. After 48 h of transfection, total RNA was prepared and analyzed for the expression of the indicated transcripts by reverse transcriptase-PCR as described. Data from (C) were quantified and normalized relative to the β-actin mRNA level using ImageJ version 1.35d (NIH Image). Data shown are representative of two independent experiments.

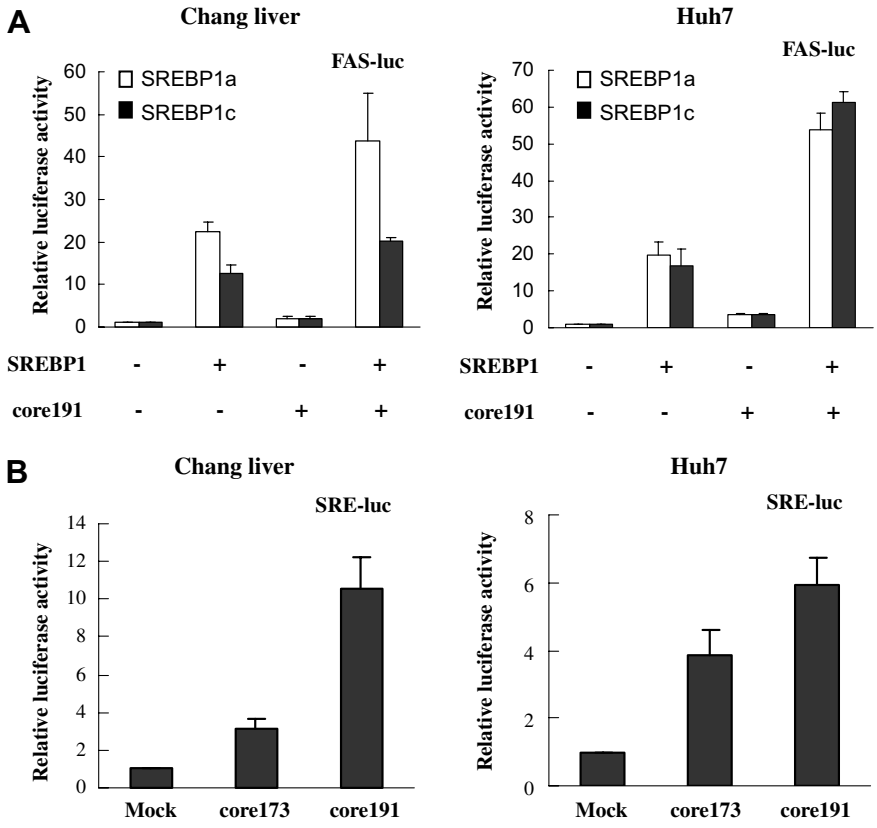

Fig. 2. The effect of HCV core protein on the transcriptional activity of SREBP1. (A) The enhanced transcriptional activity of exogenous SREBP1 by HCV core protein. Cells were transfected with indicated expression plasmids. After 48 h of transfection, cell lysates were obtained and analyzed. Data shown are means ± SD of three independent experiments performed in duplicate. (B) The increased transcriptional activity of endogenous SREBP1 by HCV core protein. Cells were transfected with indicated expression plasmids. After 48 h of transfection, cell lysates were obtained and analyzed. Data shown are means ± SD of three independent experiments performed in duplicate.

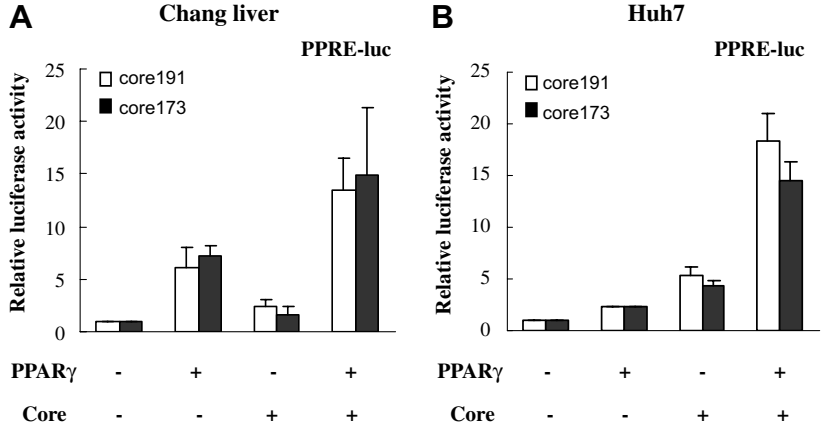

Fig. 3. The effect of HCV core protein on PPARγ transcriptional activity. (A,B) The increased PPARγ transcriptional activity by HCV core protein. Cells were transfected with indicated expression plasmids. After 48 h of transfection, cell lysates were obtained and analyzed. Data shown are means ± SD of three independent experiments performed in duplicate.

HCV core induces PPARγ transcriptional activity but not PPARγ expression

It is known that PPARγ is as a key regulator of hepatic lipid uptake and lipid synthesis. Recently, it is reported that the growing cells in delipidated medium suppressed the triglycerides accumulation induced by HCV core protein [17]. We assumed that HCV core-induced lipid deposition may be relevant to lipid uptake. Because PPARγ plays an important role in hepatic lipid uptake, we tested whether HCV core protein modulates the expression and transcriptional activity of PPARγ. HCV core protein did

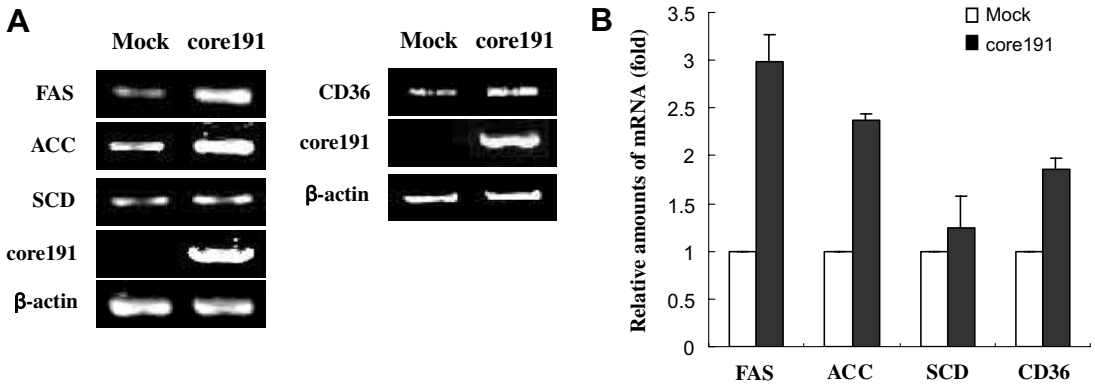

Fig. 4. The effect of HCV core protein on the expression of fatty acid synthesis and uptake associated genes. (A) Total RNA was extracted from cells transiently expressing core protein and RT-PCR was performed using the indicated primers. Data shown are representative of two independent experiments. (B) Data from (A) were quantified and normalized relative to the β-actin mRNA level using ImageJ version 1.35d (NIH Image).

not significantly affect the protein level of PPARγ in Chang liver, Huh7 and HepG2 cells, although the expression of PPARγ by HCV core protein was slightly increased in Chang liver cells (data not shown). Next, to determine the effect of HCV core protein on transcriptional activity of PPARγ, PPRE-luciferase reporter activity by transfection of PPARγ and core protein expression vectors was measured in Chang liver and Huh7 cells. As shown in Fig. 3A and B, both the full length and mature core protein upregulated the transcriptional activity of PPARγ. Taken together, these results demonstrate that HCV core protein promotes the transcriptional activity of PPARγ, but not gene expression of PPARγ.

HCV core protein stimulates the gene expression of enzymes involved in fatty acid synthesis and fatty acid uptake

Finally, we asked whether HCV core protein induces the expression of genes, which were identified as target proteins of SREBP1 and PPARγ. The mRNA expression of genes involved in fatty acid synthesis and fatty acid uptake was measured by RT-PCR in Chang liver cells expressing full length core protein (Fig. 4A and B). The mRNA expression of FAS and ACC, but not SCD was significantly increased in core-transfected cells compared with pcDNA-HA transfected cells. The gene expression of CD36, a novel target of PPARγ, was slightly increased. These data indicate that HCV core protein may induce hepatic lipid accumulation through the increase in genes expression associated with fatty acid synthesis and uptake.

Discussion

Numerous studies suggest that HCV core protein plays a pivotal role in HCV-induced liver pathogenesis through altering the expression of genes that are associated with hepatic steatosis [4,5], fibrosis [6], and malignant transformation [7]. It is reported that HCV core protein-induced steatosis is induced by the decrease of VLDL secretion [8] and by inhibition of fatty acid oxidation [9]. However, because of the various pathways of hepatic lipid metabolism, HCV core protein may induce liver steatosis through other lipid metabolic pathways. Strongly supporting the notion, recent studies indicate that HCV infection stimulates fatty acid synthetic pathway [18,19]. Moreover, HCV core-caused lipid deposition has been reported to be involved in lipid uptake [17]. However, the molecular mechanism by which HCV core protein is associated with lipid synthesis and lipid uptake, is unknown. In this report, our study offers the first evidence that HCV core protein is able to increase hepatic lipid accumulation through elevating the expression and activity of SREBP1 and PPARγ activity.

It is known that HCV requires the fatty acid biosynthetic pathway for efficient HCV RNA replication [18,19]. Here, we showed that HCV core protein may play a major role in an increase of fatty acid synthesis through SREBP1 activation. Therefore, our current data indicate the possibility that HCV core-induced hepatic lipid synthesis via SREBP1 activation may contribute to efficient HCV replication.

In general, accelerated fatty acid synthesis is showed in various tumour tissues including breast, lung, colon, prostate, and hepatocellular carcinomas (HCC) [20]. It is presumed that there is a link between cellular proliferation and fatty acid synthesis because fatty acid synthesis has been activated when cells enter into proliferative phase. Strongly supporting the notion, several studies indicate that fatty acid synthesis appears to be required for cell growth and proliferation [21]. Although the mechanisms underlying the increased lipogenic gene expression in tumors are not fully understood, it is presumed that the increased expression of lipogenic enzymes is mediated at least in part by activation of the SREBP pathway. Consistent with this hypothesis, recent study reports that SREBP1 may provide a link between lipid synthesis, proliferation, and cell growth [22]. Taken together with previous findings, our current data suggest a possibility that the increased fatty acid synthesis by HCV core protein-induced SREBP1 activation may be involved in development of HCV-induced HCC. Further experiments are required to address this interesting issue.

Recent studies have established a role for hepatic PPARγ in the development of hepatic steatosis in mouse model [13,14]. According to these studies, hepatic PPARγ is able to contribute to hepatic steatosis through increasing hepatic triglyceride production and hepatic lipid uptake. Interestingly, previous study showed that the lipid accumulation by HCV core protein may be correlated with lipid uptake [17]. In the present study, we observed that HCV core protein elevates PPARγ transcriptional activity, not gene expression, leading to upregulation of fatty acid uptake-associated gene CD36. However, one recent report suggests that the decreased level of PPARγ is associated with steatosis of HCV infected patients [23]. Therefore, although HCV core protein is correlated with the increase of PPARγ activity in vitro cell culture models, more studies are needed to evaluate whether HCV core-mediated PPARγ activation is involved in hepatic lipid accumulation.

In conclusion, we showed that HCV core protein is able to induce the gene expression and transcriptional activity of SREBP1, thereby causing the increase of fatty acid synthesis. We also observed that HCV core protein elevates PPARγ activity, thereby inducing the expression of fatty acid uptake-associated gene. Thus, our results suggest that SREBP1 and PPARγ may represent a new potential therapeutic target in the pathogenesis in HCV infection.

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