SPANDIDOS PUBLICATIONS

Changes in the expression of cholesterol metabolism-associated genes in HCV-infected liver: A novel target for therapy?

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Abstract. Recent investigations indicate that hepatitis C virus (HCV) infection is closely associated with hepatocytic lipid metabolism and induces hepatic steatosis. However, the actual lipid metabolism in HCV-infected liver has not been extensively investigated in humans. In this study, we evaluated the expression of lipid metabolism-associated genes in patients with HCV infection by real-time PCR. Sterol regulatory element-binding protein (SREBP)-2 expression was unchanged and low density lipoprotein receptor expression was markedly reduced by 90% in HCV-infected liver. The expression of apolipoprotein B100, microsomal triglyceride transfer ptotein and ATP-binding cassette G5 was significantly increased. Up-regulation of cholesterol synthesis-associated genes, including HMG-CoA reductase, HMG-CoA synthase, farnesyl-diphosphate synthase and squalene synthase, confirmed enhanced *de novo* cholesterol synthesis. The expression of cholesterol 7α-hydroxylase and farnesoid X receptor was enhanced, while bile salt export pump expression was unchanged. Fatty acid synthase expression was increased which was accompanied by increased expression of liver X receptor α and SREBP-1c. In summary, the regulation of lipid metabolism was impaired and cholesterol and fatty acid synthesis continued to increase without negative feedback in HCV-infected liver. These changes may be beneficial for HCV replication.

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Abbreviations: ABC, ATP binding cassette; BSEP, bile salt export pump; CYP7A1, cholesterol 7α-hydroxylase; EPA, eicosapentaenoic acid; FAS, fatty acid synthase; FXR, farnesoid X receptor; HMGR, HMG-CoA reductase; IFN, interferon; LDLR, low density lipoprotein receptor; LXR, liver X receptor; MTP, microsomal triglyceride transfer protein; SREBP, sterol regulatory element-binding protein; VLDL, very low density lipoprotein

Key words: hepatitis C virus, cholesterol, lipid-metabolism, chronic hepatitis, fatty acid

Introduction

A close association between hepatitis C virus (HCV) infection and lipid metabolism was previously reported. For example, the low density lipoprotein receptor (LDLR) is a target for HCV entry into hepatocytes (1,2), therefore, ß-lipoproteins influence HCV proliferation. Serum HCV-Ag levels are negatively correlated with serum ß-lipoproteins (3) and LDL-cholesterol levels are correlated with the outcome of HCV treatment with interferon (IFN) (4,5). HCV core protein induces hepatic lipid accumulation by activating sterol regulatory element-binding protein (SREBP)-1c (6,7). In addition, liver microsomal triglyceride transfer protein (MTP), a key enzyme for the assembly of very low density lipoprotein (VLDL), may be involved in HCV-related steatosis, and hepatic MTP expression and steatosis showed significant negative correlation in patients with chronic hepatitis C (8-11). Approximately 50% of patients with chronic hepatitis C have hepatic steatosis which enhances disease progression (12-14). Host metabolic factors as well as viral factors should be involved in the pathogenesis of hepatic steatosis. However, the actual lipid metabolism in HCV-infected liver has not been extensively

Table I. Primer sets of real-time PCR.

Genes	Forward (5'→3')	Reverse (5'→3')
LXRα	GCCGAGTTTGCCTTGCTCA	TCCGGAGGCTCACCAGTTTC
SREBP-1c	GCTGTCCACAAAAGCAAATCTCT	GTCAGTGTGTCCTCCACCTCAGT
SREBP-2	ACAACCCATAATATCATTGAGAAACG	TTGTGCATCTTGGCGTCTGT
LDLR	CAACGGCTCAGACGAGCAAG	AGTCACAGACGAACTGCCGAGA
BSEP	CTTCATCATGGACCTGCCACA	GGATGAGGGCTCTGGCGATA
FXR	ACCTCGACAACAAAGTCATGCAG	ATTGGTTGCCATTTCCGTCA
CYP7A1	AGCATTTGTGAATACATGGCTGGA	TTCACAAGCAAGCACTGGTGAAC
ABCG5	ATTGTGGTTCTCACCATTCACCAG	GGTTTGAATGTTCAGGACAAGGGTA
ApoB	TCAAGAGTTACAGCAGATCCATCAA	TCAGAATGGAAGTCCTTAAGAGCAA
MTP	AGCACCTCAGGACTGCGAAGA	CAGAGGTGACAGCATCCACCA
HMGR	GCCTGGCTCGAAACATCTGAA	CTGACCTGGACTGGAAACGGATA
HMGS	GTATGCCCTGGTAGTTGCAGGAG	TGTTGCATATGTGTCCCACGAA
FDPS	GCATGTATCTACCGCCTGCTGA	TCCAGGGTCTGCCCAATCTC
SS	CGTGCAGTGCCTGAATGAACTTA	GGCAGCCAAAGTGGCAATG
NPC1L1	CCCTGCCCAAGGACTCGTA	AGTTGTAGCCCAAGGTGGTAACA
FAS	GAACTCCTTGGCGGAAGAGA	GGACCCCGTGGAATGTCA
ß-actin	TGGCACCCAGCACAATGAA	CTAAGTCATAGTCCGCCTAGAAGCA

LXR, liver X receptor; SREBP, sterol regulatory element-binding protein; LDLR, LDL receptor; BSEP, bile salt export pump; FXR, farnesoid X receptor; CYP7A1, cholesterol 7α-hydroxylase; ABCG5, ATP-binding cassette G5; ApoB, apolipoprotein B; MTP, microsomal triglyceride transfer protein; HMGR, HMG-CoA reductase; HMGS, HMG-CoA synthase; FDPS, farnesyl-diphosphate synthase; SS, squalene synthase, NPC1L1, Niemann-Pick C1 like 1; FAS, fatty acid synthase.

investigated in humans. Therefore, we evaluated the expression of lipid metabolism-associated genes in HCV-infected liver.

Materials and methods

Tissue samples were obtained by liver biopsy from 70 patients (males/females, 29/41; age, 56.1±11.5 years old) with chronic hepatitis C (genotype 1b, n=45; 2a/2b, n=25), who were admitted to the Kyushu Medical Center in 2007-2008. For a control, normal liver tissue was obtained from 10 living donors of liver transplantation whose liver function tests and histological findings were completely normal. Written informed consent was obtained from all patients in this investigation. Gene expression was examined by real-time RT-PCR. The PCR primer sets are listed in Table I.

Total RNA was prepared from the tissue samples with Trizol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized from 1.0 μg RNA with GeneAmp RNA PCR (Applied Biosystems, Branchburg, NJ, USA) using random hexamers. Real-time RT-PCR was performed using LightCycler-FastStart DNA Master SYBR Green 1 (Roche, Basel, Switzerland) according to the manufacturer's instructions. The reaction mixture (20 μl) contained LightCycler-FastStart DNA Master SYBR Green 1, 4 mM $MgCl_2$, 0.5 μM of the upstream and downstream PCR primers, and 2 μl of first-strand cDNA as a template. To control variations in the reactions, all PCR data were normalized against ß-actin expression. Continuous variables were compared using the Wilcoxon signed-rank test. $P<0.05$ was considered statistically significant. The results are expressed as means ±SD.

Results

Expression levels of cholesterol metabolism-associated genes in HCV-infected liver were compared with those in normal controls. The results of real-time PCR are shown in Figs. 1 and 2. Serum LDL-cholesterol is taken into hepatocytes via the LDLR. For secretion, cholesterol is packed into VLDL together with triglyceride and apoB100 by MTP. Cholesterol is also secreted into bile via ATP binding cassette (ABC) G5/8. SREBP-2 synchronously activates the gene expression of LDLR and HMG-CoA reductase (HMGR), a key enzyme of cholesterol synthesis. Physiologically, cholesterol accumulation in hepatocytes down-regulates SREBP-2 activity, thus decreasing cholesterol synthesis/uptake. Surprisingly, in HCV-infected liver, HMGR expression was increased by >5-fold, while SREBP-2 expression was unchanged (Figs. 1 and 2). In contrast, LDLR expression was markedly reduced by 90% (Fig. 1). The expression of apoB100 and MTP was increased by >3-fold and ABCG5 expression was also increased (Fig. 1). Up-regulation of other cholesterol synthesis-associated genes, including HMG-CoA synthase, farnesyl-diphosphate synthase and squalene synthase, confirmed enhanced *de novo* cholesterol synthesis (Fig. 2). Cholesterol 7α-hydroxylase (CYP7A1) is a key enzyme involved in bile acid synthesis and its expression is negatively regulated by farnesoid X receptor (FXR). Bile acid is transported into bile by bile salt export pump (BSEP), the expression of which is positively regulated by FXR. Liver X receptor α (LXRα), whose agonists include oxysterols, up-regulates another transcriptional factor, SREBP-1c, to promote fatty acid production via fatty acid synthase (FAS). In the HCV-infected liver, CYP7A1 expression

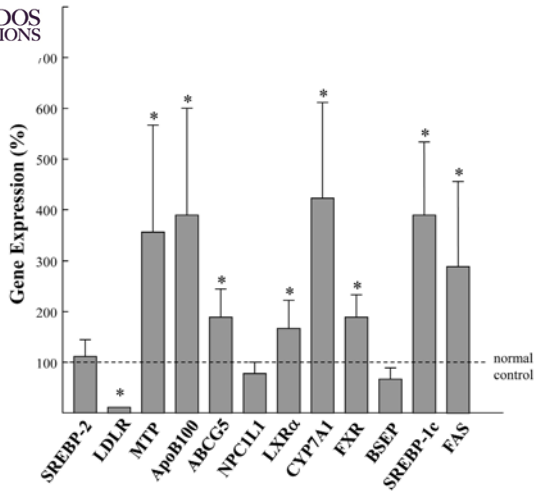

Figure 1. Expression levels of cholesterol metabolism-associated genes in HCV-infected liver. SREBP, sterol regulatory element-binding protein; LDLR, LDL receptor; MTP, microsomal triglyceride protein; ApoB, apolipoprotein B; ABCG5, ATP-binding cassette G5; NPC1L1, Niemann-Pick C1 like 1; LXRα, liver X receptor α; CYP7A1, cholesterol 7α-hydroxylase; FXR, farnesoid X receptor; BSEP, bile salt export pump; FAS, fatty acid synthase. *Significant difference ($p<0.05$) between patients with chronic hepatitis C and normal controls.

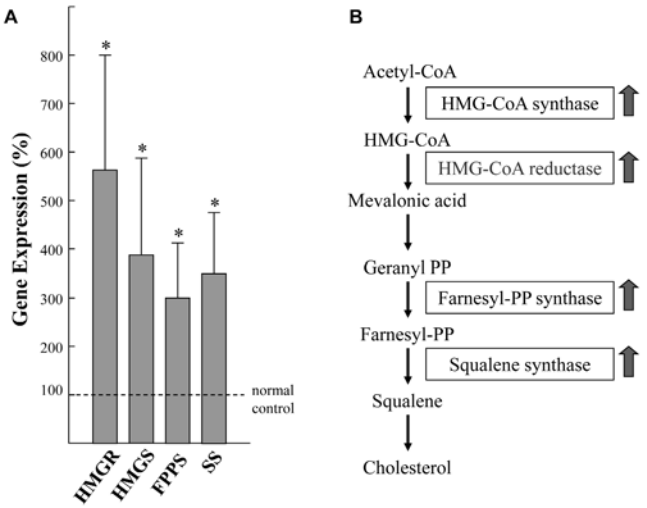

Figure 2. (A) Real-time RT-PCR analysis of HMG-CoA reductase (HMGR), HMG-CoA synthase (HMGS), farnesyl-diphosphate synthase (FDPS), and squalene synthase (SS) gene expression in HCV-infected liver. *Statistically significant differences ($p<0.05$) compared with normal liver (100%). (B) Cholesterol synthesis pathway in hepatocytes and its related enzymes. Arrows indicate significant upregulation of expression levels in HCV-infected liver compared with normal control.

was enhanced by 4-fold, which was accompanied by increased expression of FXR, while BSEP expression was unchanged (Fig. 1). FAS expression was increased by ~3-fold and was accompanied by increased expression of LXRα and SREBP-1c (Fig. 1).

Discussion

Expression pattern of examined lipid metabolism-associated genes in the liver of chronic hepatitis C is summarized in Fig. 3. In our investigation, the regulation of lipid metabolism was impaired in HCV-infected liver. It is probable that HCV infection induces intra-hepatic accumulation of cholesterol, which results in decreased LDL-cholesterol uptake and increased lipoprotein and cholesterol output. Nevertheless, *de novo* cholesterol synthesis and fatty acid synthesis continued to increase without negative feedback (Fig. 2). We cannot explain the phenomena clearly but the same discrepancy was found in nonalcoholic fatty liver disease (15). The expression patterns of the tested genes were also apparent in a preliminary evaluation in an HCV replicon system (data not shown).

These changes seem to be needed or are beneficial for HCV replication. Considering the enhanced cholesterol synthesis in HCV-infected liver, it is plausible that HMGR inhibitors (statins) elicit inhibitory effects on viral replication. Statins were recently reported to suppress HCV replication and, in a clinical trial on peg-IFN plus ribavirin combination therapy, fluvastatin showed synergistic antiviral effects (16,17). In addition, geranylgeranyl-diphosphate and farnesyl-diphosphate, which are produced through the *de novo* cholesterol synthesis pathway, are reported to be essential for viral replication (18). They are needed to activate small GTPases such as Rho and Ras, therefore, HCV may need lipids not only for components of virus particles but also for the modulation of cell signaling pathways. It is also expected

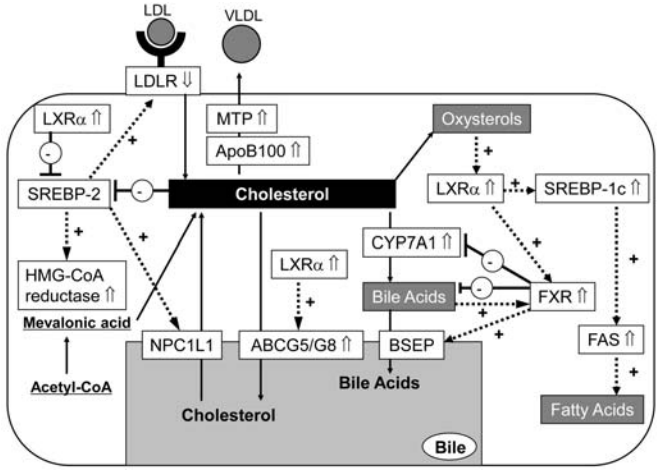

Figure 3. Schema showing the interactions between cholesterol metabolism-associated factors. Arrows (⇑ and ⇓) represent significant difference of expression levels between patients with HCV infection and normal controls. LDLR, LDL receptor; SREBP, sterol regulatory element-binding protein; NPC1L1, Niemann-Pick C1 like 1; ApoB, apolipoprotein B; MTP, microsomal triglyceride protein; ABCG5/G8, ATP-binding cassette G5/G8; LXRα, liver X receptor α; CYP7A1, cholesterol 7α-hydroxylase; FXR, farnesoid X receptor; BSEP, bile salt export pump; FAS, fatty acid synthase.

that bisphosphonate has antiviral effects because bisphosphonate inhibits farnesyl-diphosphate synthase, the expression of which was enhanced in HCV-infected liver. Furthermore, eicosapentaenoic acid (EPA) was reported to inhibit HCV replication in the replicon system and to suppress SREBP-1c activity (19,20). Therefore, EPA might elicit antiviral effects via the inhibition of SREBP-1c. We are now performing a clinical trial using the lipid modulators, statins, bisphosphonate and/or EPA, in combination with peg-IFN plus ribavirin therapy.

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