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Increased serum oxysterol concentrations in patients with chronic hepatitis C virus infection

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ABSTRACT

Oxidative stress and dysregulated cholesterol metabolism are characteristic features of chronic hepatitis C virus infection (CHC). Therefore, we analyzed serum oxysterol profiles in CHC patients and examined the significance of oxysterols in CHC. The concentrations of 7α-hydroxycholesterol, 4β-hydroxycholesterol and 25-hydroxycholesterol as determined by LC–ESI–MS/MS were significantly elevated by +236%, +29% and +44%, respectively, in CHC patients compared with controls. Moreover, the elevated levels were significantly decreased by anti-viral therapy using PEGylated-interferon and ribavirin for 3 months. In contrast, 24S-hydroxycholesterol, 27-hydroxycholesterol and 7α-hydroxy-4-cholesten-3-one concentrations were not affected by CHC or anti-viral treatment. These results suggest that some oxysterols that are elevated in CHC are produced by cholesterol autoxidation due to oxidative stress or inflammation in the liver. Oxysterols may represent novel targets for the inhibition of disease progression and the prevention of hepatocarcinogenesis in CHC patients.

1. Introduction

Oxysterols can be derived from the diet or from endogenous cellular biosynthesis, and they play important roles in physiological and pathological processes. The pathogenic effects of oxysterols have been described predominantly in cardiovascular diseases [1–3], degenerative diseases such as age-related macular degeneration [4], Alzheimer's disease [5,6] and osteoporosis [7,8]. However, recent reports have focused on the roles of oxysterols in carcinogenesis [9,10].

Chronic hepatitis C virus infection (CHC) is a primary risk factor for liver cancer. Progression of CHC and carcinogenesis are thought to be associated with oxidative stress and the dysregulation of lipid metabolism in the liver [11,12]. Yoshida et al. quantified oxysterols in human hepatic bile with bacterial infection and revealed a positive correlation between the biliary concentrations of several oxysterols and serum C-reactive protein (CRP) levels [13]. The authors speculated that reactive oxygen species (ROS) derived from activated leukocytes produced the oxysterols non-enzymatically. Another group reported a significant increase in hepatic oxysterol levels in a hamster model of cholangiocarcinoma induced by liver fluke infection [10], suggesting that long-term exposure to oxysterols may contribute to the progression of inflammation and carcinogenesis.

We previously reported the elevation of serum oxysterol concentrations in patients with non-alcoholic fatty liver disease (NAFLD), and a total serum oxysterol level associated with insulin resistance [14]. In addition, we also demonstrated that therapeutic intervention, i.e., the administration of statins, resulted in a gradual reduction in serum oxysterol levels in NAFLD patients [14]. In the present study, we determined the serum oxysterol concentrations in patients with CHC and compared the levels with those in sera obtained from healthy volunteers. The effects of anti-viral therapy on serum oxysterol concentrations were also studied. Our results demonstrate that CHC patients also exhibit hyperoxysterolemia and that this elevation was suppressed by anti-viral therapy.

2. Subjects, materials and methods

2.1. Subjects

In this case-control study, we evaluated patients diagnosed with CHC at the National Hospital Organization Kyushu Medical

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Center. For the use of serum samples for the study purpose, written informed consent was obtained from each patient. In all patients, current and past daily alcohol consumption was less than 20 g per week. Exclusion criteria other than excessive alcohol consumption were as follows: evidence of pregnancy, treatment with corticosteroids, and hormone replacement therapy. Subjects using lipid-lowering medications or food enriched with functional plant sterols or stanols were excluded from the study. Subjects with positive test results for the following disorders were also excluded: drug-induced liver injury, autoimmune hepatitis, primary biliary cirrhosis, alpha-1-antitrypsin deficiency, hemochromatosis, Wilson's disease, and biliary obstruction. Venous blood samples were collected in the morning (following a 12-h overnight fast) at baseline and 3 months after the initiation of combination treatment with PEGylated-interferon (PEG-IFN; Pegasys®, Chugai Pharmaceutical Co., Tokyo, or Peg-Intron®, MSD K.K, Tokyo) and ribavirin (RBV; Copegas® Chugai Pharmaceutical Co., Tokyo, or Rebetol®, MSD K.K, Tokyo). Serum samples were stored at −20 °C until later analysis.

Fasting sera of 113 age- and sex-matched healthy volunteers without obesity, hyperlipidemia, diabetes or liver dysfunction (obtained for another study group [courtesy of Professor T. Teramoto, Teikyo University, with written informed consent from the healthy volunteers] were obtained and used as the control group. The control serum samples were stored and handled as mentioned above.

2.2. Quantification of serum lipid biomarkers

Serum oxysterols, including 24S-hydroxycholesterol (24SHC), 25-hydroxycholesterol (25HC), 27-hydroxycholesterol (27HC), 7α-hydroxycholesterol (7αHC), 4β-hydroxycholesterol (4βHC), 22R-hydroxycholesterol (22RHC) and 24S,25-epoxycholesterol, were quantified by LC–MS/MS as described in our previous papers [15–17]. Briefly, coprostanol and deuterated oxysterols were added to 10 μL of serum as internal standards, and alkaline hydrolysis was performed in 1 N ethanolic KOH with butylated hydroxytoluene at 37 °C for 1 h. Sterols were extracted with *n*-hexene, derivatized to picolinyl esters, and injected into the LC–ESI–MS/MS system, which consisted of a TSQ Vantage triple stage quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with an HESI-II probe and a Prominence ultra-fast liquid chromatography (UFLC) system (Shimadzu, Kyoto, Japan).

The serum concentrations of 7α-hydroxy-4-cholesten-3-one (C4), a biomarker of CYP7A1 activity, were determined by LC–MS/MS without alkaline hydrolysis [18]. Deuterium-labeled C4 was added to 20 μL of serum, and C4 was extracted with acetonitrile. After derivatization into the picolinyl ester, C4 was analyzed by the LC–ESI–MS/MS system described above.

All samples were thawed for immediate analysis and were not reused. For purification, all samples were maintained under nitrogen to avoid autoxidation during the assays.

2.3. Statistical analysis

Statistical analyses were performed either by one-way analysis of variance or the two-tailed Student *t* test using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). The results are presented as the mean ± SEM, and *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Characteristics of study subjects

Sera obtained from a total of 55 CHC patients and 113 healthy controls were used for the present study. The clinical characteristics of the subjects are presented in Table 1. The control subjects were selected after matching for age and sex. After selection, the mean age and male/female ratio did not differ significantly between the control and CHC groups. In the control group, none of the subjects were obese (BMI > 25), nor did any exhibit hypercholesterolemia (total cholesterol > 220 mg/dl), hypertriglyceridemia (TG > 150 mg/dl), hypertension or diabetes. However, in the CHC group, there were three patients with hypertension and three patients with diabetes, and eight of the 55 CHC patients exhibited obesity (BMI > 25). In the CHC group, 32 patients were infected with viral genotype 1b, 13 with genotype 2a, seven with genotype 2b and three with an unknown genotype. Among 32 patients in the CHC group, ultrasound-guided percutaneous liver biopsy was performed before the initiation of the treatment. Pathological finding was determined by two independent pathologists, and the progression of fibrosis was defined according to the New-Inuyama classification [19]. The fibrosis stage was defined as F1 in 20 patients, F2 in nine patients and F3 in three patients.

3.2. Serum oxysterol levels in CHC patients

As shown in Table 1, the total serum cholesterol concentrations were significantly lower in patients with CHC. Likewise, HDL-cholesterol and LDL-cholesterol were also significantly lower in CHC patients compared with controls. The concentrations of 4βHC, 25HC and 7αHC were significantly elevated by +29%, +44% and +236% in CHC patients compared with controls, respectively. In contrast, 24SHC and 27HC concentrations in CHC patients were not significantly different from those in controls. We also analyzed

Table 1
Serum cholesterol and oxysterol concentrations in healthy controls and patients with chronic hepatitis C virus infection.

Background and sterol data	Controls (*n* = 113)	CHC patients (*n* = 55)	*P*
Gender (male/female)	53/60	22/33	N.S.
Age (years)	52.3 ± 1.4	57.4 ± 1.6	N.S.
Total cholesterol (mg/dl)	191.2 ± 3.3	167.7 ± 4.8	<0.0005
LDL-cholesterol (mg/dl)	108.3 ± 2.9	97.4 ± 3.8	<0.05
HDL-cholesterol (mg/dl)	66.5 ± 1.5	53.4 ± 2.4	<0.0005
4β-Hydroxycholesterol (ng/ml)	51.9 ± 2.4	66.9 ± 2.8	<0.0001
25-Hydroxycholesterol (ng/ml)	14.9 ± 1.1	21.4 ± 0.8	<0.001
24S-Hydroxycholesterol (ng/ml)	64.4 ± 1.8	64.6 ± 2.0	N.S.
27-Hydroxycholesterol (ng/ml)	139.0 ± 4.7	138.6 ± 6.3	N.S.
7α-Hydroxycholesterol (ng/ml)	136.5 ± 12.0	458.6 ± 18.6	<0.0001
C4 (ng/ml)	27.5 ± 3.0	26.3 ± 2.4	N.S.
C4 (ng/mg total cholesterol)	14.7 ± 1.6	12.7 ± 1.4	N.S.

The data are expressed as the mean ± SEM.
CHC, chronic hepatitis C virus infection; C4, 7α-hydroxy-4-cholesten-3-one; N.S., not significant.

22RHC and 24S,25-epoxycholesterol, but only trace amounts were detected by our present method. The serum concentration of C4 (7α-hydroxy-4-cholesten-3-one), a marker oxysterol for CYP7A1 activity, was also measured. Absolute concentrations as well as the levels relative to total cholesterol concentrations did not significantly differ between the CHC patients and controls. These oxysterol concentrations were comparable among the subgroups sorted by viral genotype or fibrosis stage. However, no significant difference was observed among the subgroups.

3.3. Effects of early-phase anti-viral therapy on serum oxysterol levels

To determine the effects early-phase anti-viral therapy on oxysterol levels, serum samples obtained from 41 CHC patients before and 3 months after treatment with PEG-IFN/RBV were analyzed. Among 41 patients, 30 achieved early virological response (EVR), i.e., levels of plasma viral RNA below detectable levels (by real-time PCR), within 3 months of the initiation of treatment. Although the other patients did not achieve EVR, significantly reduced levels of plasma viral RNA were also observed. After 3 months of the treatment, the total serum cholesterol levels tended to decrease (150.2 ± 4.1 mg/dl) compared with pre-treatment levels (167.7 ± 4.8 mg/dl), although the difference was not statistically significant. As shown in Fig. 1, the absolute concentrations of 4βHC, 25HC and 7αHC as well as the levels relative to the total cholesterol concentrations were significantly reduced by PEG-IFN/RBV treatment but remained higher than those of control subjects. In particular, the change in serum 7αHC levels before and after PEG-IFN/RBV therapy was significant. In contrast, the serum concentrations of 24SHC, 27HC and C4 were not affected by PEG-IFN/RBV treatment. Furthermore, there was no significant difference in oxysterol levels between the EVR group and the non-EVR group (data not shown), suggesting that viral eradication is unnecessary to promote normalization of serum oxysterol levels.

4. Discussion

It is well established that serum cholesterol levels are decreased in patients with CHC and that cholesterol levels return to normal after the eradication of the virus. HCV virus blocks cholesterol release from hepatocytes by disturbing VLDL secretion [20] to utilize excess cholesterol to sustain its own life cycle. Miyazaki et al. recently analyzed public health examination data from 146,857 Japanese people and demonstrated that serum lipid concentrations were significantly lower in asymptomatic HCV carriers with normal aminotransferase levels (ALT < 30 and AST < 30) [21]. In addition, our preliminary data of serum biomarker sterol concentrations suggest that endogenous cholesterol biosynthesis is not up-regulated despite of the presence of hypocholesterolemia in CHC patients (Ikegami, manuscript in preparation).

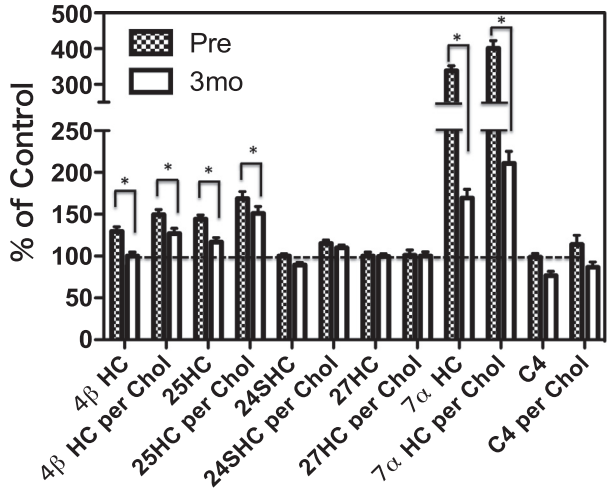

Fig. 1. Change of serum oxysterol levels by PEG-IFN/RBV treatment. Each serum oxysterol level before (Pre) and 3 months after initiation of PEG-IFN/RBV therapy (3 months) was indicated as percent of control (mean of control subjects). 4βHC: 4β-hydroxycholesterol, 25HC: 25-hydroxycholesterol, 24SHC: 24S-hydroxycholesterol, 27HC: 27-hydroxycholesterol, 7αHC: 7α-hydroxycholesterol, C4: 7α-hydroxy-4-cholesten-3-one "per Chol" represents values relative to serum cholesterol level. *$P < 0.01$.

Oxysterols are oxygenated metabolites of cholesterol that are short-lived intermediates in cholesterol excretion pathways [22]. Oxysterols are present in very low concentrations in mammalian systems, always accompanied by an excess of cholesterol [23]. Oxysterols have been identified not only as potent ligands of liver X receptor, but also as important players in physiological and pathological processes, including carcinogenesis [24] and atherosclerosis [2]. CHC is a primary risk factor for liver cancer. The striking findings of the present study were the significant elevation of serum oxysterol concentrations in patients with CHC despite hypocholesterolemia and the reduction of oxysterol levels during antiviral therapy (PEG-IFN/RBV). Although the direct effects of elevated oxysterol concentrations in CHC on hepatocarcinogenesis remain unknown, oxysterols may be potential biomarkers for the evaluation of cancer risk.

Oxysterols are derived from either enzymatic or non-enzymatic oxidation of cholesterol. The enzymatic production of 4βHC, 24SHC, 27HC and 7αHC involves the cytochrome P450 family enzymes CYP3A4, CYP46A1, CYP27A1 and CYP7A1, respectively. Another oxysterol, 25HC, is synthesized by CYP3A4, CYP46A1, CYP27A1 and a non-cytochrome P450 enzyme, cholesterol 25-hydroxylase (CH25H) [25]. Furthermore, certain oxysterols are produced by non-enzymatic autoxidation, a process that involves reactive oxygen and nitrogen species (ROS, RNS). Previous reports demonstrated that 7-oxocholesterol, 7β-hydroxycholesterol (7βHC) and 7αHC are major autoxidation products of cholesterol, whereas 4βHC and 25HC are minor products [26,27]. The C-7 position of the steroid ring is known to be highly susceptible to autoxidation [28], and a repeated freeze–thaw process of sera could further increase the amount of 7αHC in our preliminary experiments. Therefore, handling, including the storage conditions of the sera determined in the present study, was highly standardized so that differences caused by sample handling would be negligible. Our results demonstrated that the absolute concentrations of 4βHC, 25HC and especially 7αHC were significantly higher in CHC patients compared with controls (Table 1), which suggests that oxidative stress caused by sustained inflammation in CHC [11,12] may enhance the autoxidation of cholesterol. Recently, Arciello et al. reported elevated concentrations of serum 7-ketocholesterol and 7βHC in CHC patients [29]. Although we did not measure these oxysterol concentrations, the results lend support to our contention that increased autoxidation in CHC patients is not a sporadic finding.

Unlike 7-ketocholesterol and 7βHC, 7αHC is produced not only by autoxidation but also enzymatically. Because CYP7A1 is the rate-limiting enzyme in the classic bile acid biosynthetic pathway, it may be possible that the elevation of 7αHC in CHC patients is due to increased bile acid synthesis. To exclude this possibility, we quantified the serum levels of C4, an immediate enzymatic product of 7αHC that reflects CYP7A1 activity without significant effects of autoxidation [18,30]. As most plasma oxysterols are observed in the LDL and HDL fractions, the oxysterols are transported in the plasma with cholesterol. Therefore, plasma C4 levels expressed relative to cholesterol are thought to be a more accurate marker for CYP7A1 activity than the absolute C4 concentrations [31]. Our

results showed that both the absolute concentrations and the relative levels of C4 did not significantly differ between control and CHC patients, which suggests unchanged hepatic bile acid synthesis in CHC patients.

Both 4βHC and 25HC are synthesized by CYP3A4 [25], and the up-regulation of this enzyme could increase the serum concentrations of these oxysterols in CHC patients. However, several reports have demonstrated that CYP3A4 activity is downregulated in CHC patients [32,33]. Therefore, it is suggested that the elevation of 4βHC and 25HC in CHC patients is also caused by cholesterol autoxidation.

The absolute concentrations of 4βHC, 25HC and 7αHC and the levels relative to total cholesterol concentrations were significantly reduced after 3 months of treatment with PEG-IFN/RBV (Fig. 1). Because C4 levels before and after PEG-IFN/RBV treatment did not change significantly, the reduction of serum 7αHC levels by PEG-IFN/RBV therapy does not appear to be due to the inhibition of CYP7A1. With respect to CYP3A4, *in vitro* and human studies demonstrated no inhibitory effects of PEG-IFN [34] or RBV (information released from pharmaceutical company, issued by KEGG MEDICUS) on CYP3A4 activity. Taken together, the reduction of serum 7αHC, 4βHC and 25HC concentrations does not seem to be associated with the pharmacologic action of PEG-IFN/RBV. Recovery from hepatic inflammation due to the suppression or eradication of the virus seems to inhibit the production of ROS from activated leukocytes. Although the exact mechanisms responsible for the dynamic alteration of these oxysterol concentrations in CHC patients remain unknown, it is possible that the degree of inflammation is related to the activity of autoxidation [13].

The activation of Toll-like receptors markedly induces CH25H and increases 25HC concentrations in mice macrophages and sera [35,36]. CH25H is reported to be an IFN-stimulated gene (ISG) conserved across mammalian species, and a recent report demonstrated that 25HC broadly inhibits viral entry by blocking membrane fusion between the virus and the cell [37]. Therefore, the elevation of serum 25HC observed in the present study may represent enhanced anti-viral activity against HCV infection in patients. However, this hypothesis is controversial for the following reasons: First, because these previous reports employed bone marrow-derived macrophages as an experimental system, it is unknown whether the same process occurs in hepatocytes, the predominant site of HCV infection. Second, in comparison with mice, the expression of CH25H has been reported to be very low in human tissues [38]. Third, the observation in the present study that IFN treatment decreased 25HC level seems to be contradictory to the previous reports proposing IFN-induced up-regulation of CH25H. By the present study, it is difficult to prove a direct association between CH25H and anti-viral action, and further study is needed to elucidate this issue.

In conclusion, the concentrations of serum oxysterols, especially 7α-HC, 25-HC and 4β-HC, were significantly elevated in CHC patients and were decreased by anti-viral therapy. Oxysterols can be novel targets for the inhibition of disease progression and the prevention of hepatocarcinogenesis in CHC patients.

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