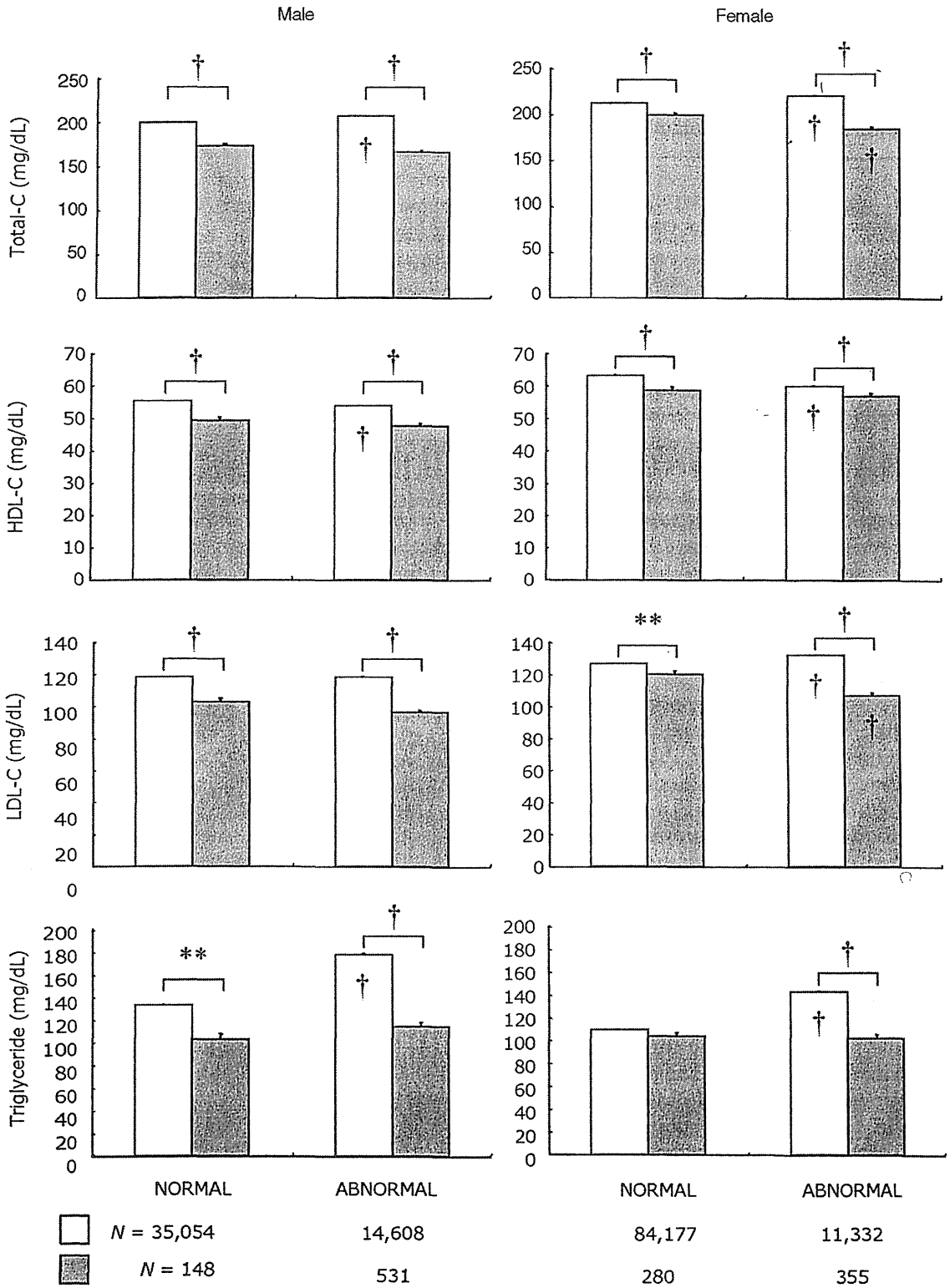

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Figure 3 Serum lipid levels classified by the healthy limits of serum aminotransferases in the hepatitis C virus (HCV) (□) negative and (▨) positive subjects. The "normal" and "abnormal" populations were classified by cut-off points: alanine aminotransferase (ALT) ≤30 and aspartate aminotransferase (AST) ≤30, and ALT >30 and/or AST >30, respectively. Data are expressed the mean ± standard error. ANOVA *P*-value was <0.0001 for all lipid parameters in both sexes. $**P < 0.01$, $\dagger P < 0.001$ by Bonferroni's post-hoc test. The symbols inside the columns of abnormal without the bar indicate the comparison against the respective normal. HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; Total-C, total cholesterol.

a significantly large number of asymptomatic HCV positive subjects with normal aminotransferase levels. In general, serum lipid levels are influenced by some factors including sex, age, diseases and/or nutritional states. As shown in Table 2, the multivariate analysis showed that most factors were significantly associated with hypolipidemia, and especially the HCV positive was the strongest factor. In comparison by matching the respective factor, serum levels of all examined lipids (Total-C, HDL-C, LDL-C and TG) were significantly decreased in the HCV positive compared to those in the HCV negative subjects, regardless of sex, age, BMI or serum aminotransferase levels. Furthermore, the significant hypolipidemia was observed in the HCV positive subjects when compared to those of the HCV negative subjects with a prior infection. Particularly, to our knowledge, the hypolipidemia in the HCV positive subjects with normal serum aminotransferase levels have never been reported.

It has been well known that the hypolipidemia caused by impaired liver function is observed in chronic liver diseases including liver cirrhosis.[20,21] Therefore, there is an apprehension whether some cirrhotic patients with lower aminotransferase levels were included in the normal population in the present study or not. In active hepatitis infected with HCV shifting to cirrhosis, both aminotransferase levels tend to decline, but are still above the normal range.[32–34] Accordingly, we assumed that there might be few chronic cirrhotic patients with HCV in the normal population. In addition, the malnutrition is generally found in the chronic cirrhotic patients, and consequently BMI would be lower. However, in the present study, the lower lipid levels were observed in all BMI classes among the HCV positive subjects. These results support the idea that the lipid abnormalities in the HCV positive subjects are directly caused by HCV infection itself rather than by the secondary effects of HCV infection, namely, hepatic damage or nutritional disorder.

Previously, some studies showed that serum LDL-C level was significantly decreased in the patients infected with HCV compared with that in the uninfected subjects.[12,14,17,35] However, serum HDL-C level was unchanged in the HCV positive subjects.[12,14,17,35] In

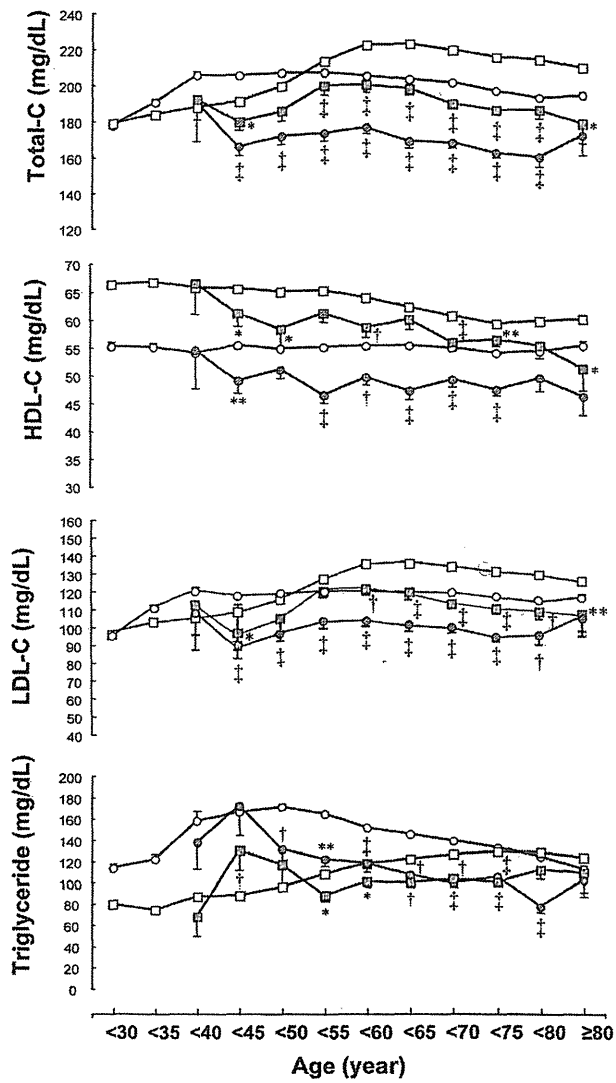

Figure 4 Serum lipid levels stratified by age ranges in the hepatitis C virus (HCV) negative and positive subjects. Data are expressed as the mean ± standard error, and the age ranges were divided into 5-year increments. Significant difference was analyzed by Mann–Whitney *U*-test between the HCV negative and positive subjects in each age-range; $*P < 0.05$, $**P < 0.01$, $\dagger P < 0.001$, $\ddagger P < 0.0001$. HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; Total-C, total cholesterol. (♂) HCV-negative: male; (♂) HCV-positive: male; (♀) HCV-negative: female; (♀) HCV-positive: female.

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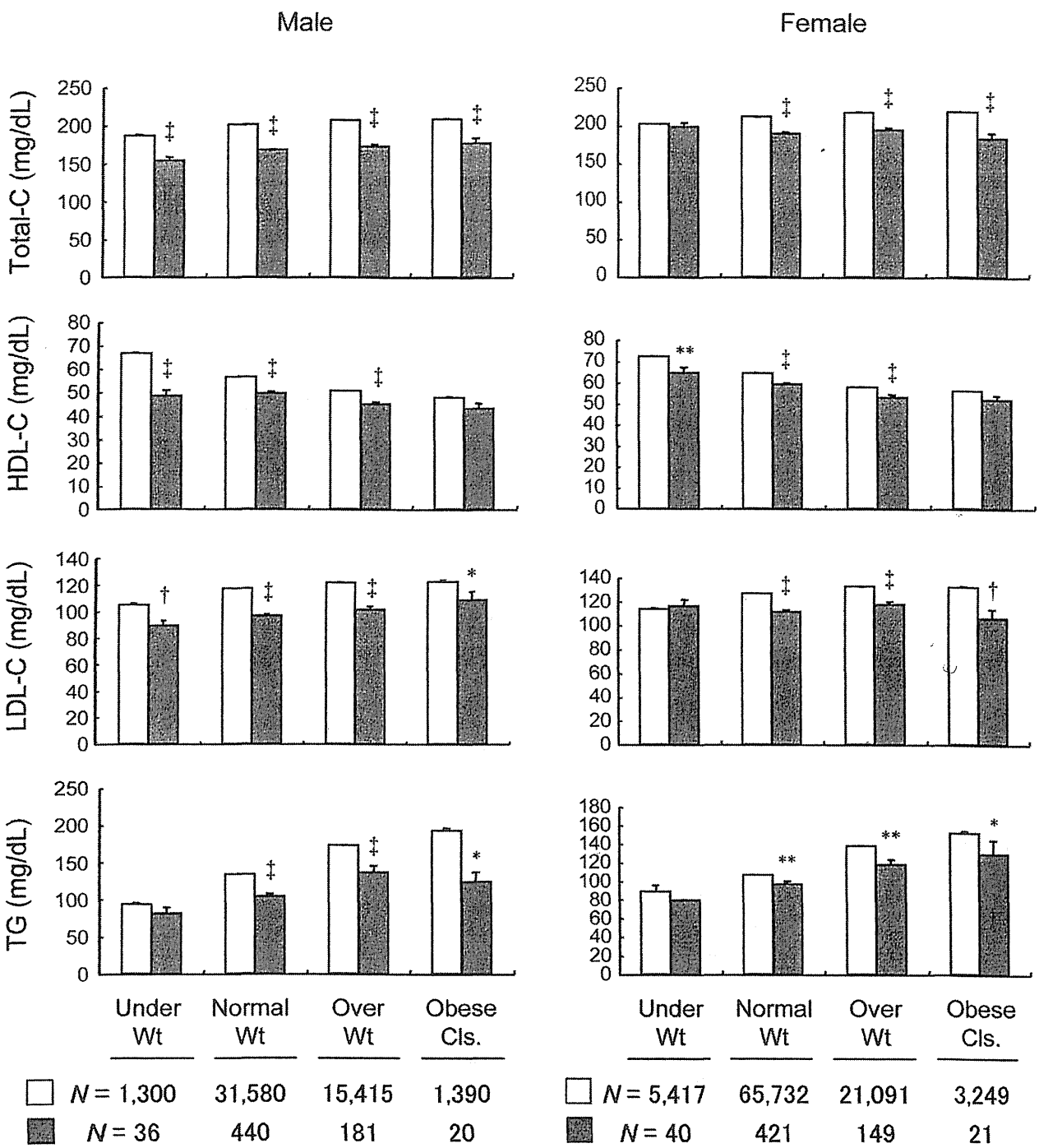

Figure 5 Serum lipid levels classified by the World Health Organization defined body mass index (BMI) class in the hepatitis C virus (HCV) (□) negative and (▩) positive subjects. Data are expressed as the mean ± standard error. Under Wt, underweight (BMI <18.5); Normal Wt, normal range of weight (18.5 ≤ BMI < 25); Over Wt, overweight (25 ≤ BMI < 30); Obese Cls, obese classes 1–3 (BMI ≥30). Significant difference between the HCV negative and positive was analyzed by Mann–Whitney *U*-test; $*P < 0.05$, $**P < 0.01$, $\dagger P < 0.001$, $\ddagger P < 0.0001$. HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; Total-C, total cholesterol.

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contrast, Siagris *et al.*[15] as well as ourselves, showed that both LDL-C and HDL-C levels were significantly reduced in the HCV positive subjects. The different findings about serum HDL-C level in the HCV positive subjects should be due to the difference in the compared control levels. In the aforementioned studies,[12,17,35] the HDL-C levels of controls were 45–47 mg/dL, which was considerably lower than those in the studies of ourselves and Siagris *et al.* (53–54 mg/dL).[15] The difference in the HDL-C level in the controls may be due to the population characteristics, including race, dietary culture and lifestyle. Thus, both HDL-C and LDL-C levels would be decreased in the HCV positive subjects who had relatively higher level of HDL-C.

In comparison between presence and absence of HCV infection, different results in serum TG level have been reported. Dai *et al.* showed the significant decrease of TG level in the HCV positive subjects in a large cohort study.[13] In contrast, there are no significant differences in serum TG level between the HCV infected patients and healthy controls in a relatively younger population (42.0 ± 14.6 years of age).[15] In the present study, we also observed the significant decreases in the TG level, but there was no difference in case of comparison in the relatively younger populations (35–44 years in males, 35–49 years in females, Fig. 4). It is not clear why serum TG level in younger ages would hardly be affected by HCV infection, and further studies are needed.

Furthermore, there are findings that genotypes of HCV are related to the reduction of hepatic lipid metabolisms. In US, Greek, Austrian, African and French patients with HCV genotype 3a, hypocholesterolemia was more remarkable than other genotypes.[14,15,36–38] Furthermore, in Egyptian patients, a significantly lower level of lipids has been also reported in HCV patients predominantly infected with genotype 4.[17] Although the HCV genotype was not determined in the present study because of cohort study in the public health examination, the most common genotypes in the Japanese population are 1b and 2a, while genotypes 3a and 4 are very rare.[39] This genotype population in Japanese is similar to the genotype populations in Taiwan where a lower level of lipids in the HCV carriers has also been reported in a cohort study.[40] Therefore, the abnormalities of serum lipids in the HCV carriers would not depend on the virus genotype.

Several previous studies have reported a relationship between lipid levels and the sustained viral response (SVR) of antivirus therapy in the HCV patients. Corey *et al.* observed that serum Total-C and LDL-C levels were significantly higher after treatment of peginterferon and ribavirin for approximately 7 months in the HCV patients with SVR compared to those in the non-responder/relapsers whose serum lipid levels did not differ from responder before the initiation of the HCV therapy.[14] Furthermore, Gopal *et al.* showed that HCV patients with higher LDL-C level before HCV therapy were associated with greater odds of achieving an SVR.[41] Therefore, focusing on the lipid prolife in the HCV patients should have important implications in the antivirus therapy including interferon and ribavirin.

Although the exact reason for the significant decrease of serum lipid levels in the HCV positive subjects is still unclear, previous studies showed HCV impaired assembly and secretion of very low-density lipoprotein from hepatocytes,[42] and reduced transport of lipids by HCV-induced oxidative stress and peroxisome proliferator-activated receptor-α inability.[43,44] In addition, a study of cholesterol metabolism by comprehensive analysis of serum biomarker sterols[45] has suggested that endogenous cholesterol biosynthesis is downregulated while intestinal cholesterol absorption is not reduced in patients with HCV infection.[46] Because lower serum cholesterol concentrations in the HCV patients could not be explained by hepatic damage or malnutrition, HCV itself might downregulate cholesterol biosynthesis in the human body.

In conclusion, the present study demonstrated that the serum levels of lipids including Total-C, LDL-C, HDL-C and TG were significantly lower in the HCV positive subjects than in the negative ones, irrespective of host factors including aminotransferase levels and nutritional states. Therefore, HCV infection itself might directly cause abnormalities of lipid metabolism.

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Cholesterol 25-hydroxylation activity of CYP3A

Akira Honda,*,†,§ Teruo Miyazaki,†,§ Tadashi Ikegami,* Junichi Iwamoto,* Tomomi Maeda,** Takeshi Hirayama,* Yoshifumi Saito,* Tamio Teramoto,** and Yasushi Matsuzaki[1],*,§

Department of Gastroenterology,* Center for Collaborative Research,† and Department of Development for Community Medicine,§ Tokyo Medical University Ibaraki Medical Center, Ibaraki 300-0395, Japan; and Department of Internal Medicine,** Teikyo University School of Medicine, Tokyo 173-8605, Japan

Abstract **To date, many studies have been conducted using 25-hydroxycholesterol, which is a potent regulator of lipid metabolism. However, the origins of this oxysterol have not been entirely elucidated. Cholesterol 25-hydroxylase is one of the enzymes responsible for the metabolism of 25-hydroxycholesterol, but the expression of this enzyme is very low in humans. This oxysterol is also synthesized by sterol 27-hydroxylase (CYP27A1) and cholesterol 24-hydroxylase (CYP46A1), but it is only a minor product of these enzymes. We now report that CYP3A synthesizes a significant amount of 25-hydroxycholesterol and may participate in the regulation of lipid metabolism. Induction of CYP3A by pregnenolone-16α-carbonitrile caused the accumulation of 25-hydroxycholesterol in a cell line derived from mouse liver. Furthermore, treatment of the cells with troleandomycin, a specific inhibitor of CYP3A, significantly reduced cellular 25-hydroxycholesterol concentrations. In cells that overexpressed human recombinant CYP3A4, the activity of cholesterol 25-hydroxylation was found to be higher than that of cholesterol 4β-hydroxylation, a known marker activity of CYP3A4. In addition, 25-hydroxycholesterol concentrations in normal human sera correlated positively with the levels of 4β-hydroxycholesterol ($r = 0.650$, $P < 0.0001$, n = 78), but did not significantly correlate with the levels of 27-hydroxycholesterol or 24*S*-hydroxycholesterol. These results demonstrate the significance of CYP3A on the production of 25-hydroxycholesterol.**—Honda, A., T. Miyazaki, T. Ikegami, J. Iwamoto, T. Maeda, T. Hirayama, Y. Saito, T. Teramoto, and Y. Matsuzaki. **Cholesterol 25-hydroxylation activity of CYP3A.** ***J. Lipid Res.*** **2011. 52: 1509–1516.**

Supplementary key words cerebrotendinous xanthomatosis • cholesterol 25-hydroxylase • CYP3A4 • CYP27A1 • CYP46A1 • 4β-hydroxycholesterol • 25-hydroxycholesterol • oxysterols

Oxysterols are physiological regulators of cellular cholesterol homeostasis (1). They downregulate HMG-CoA reductase (2–4), the rate-limiting enzyme in the cholesterol biosynthetic pathway, by blocking processing of the sterol-regulatory element binding protein (SREBP) by inducing binding of SREBP cleavage-activating protein to a protein called Insig (insulin-induced gene) (5, 6). Furthermore, there is growing evidence that certain oxysterols may accelerate ubiquitination and degradation of HMG-CoA reductase protein (1, 7). On the other hand, oxysterols are endogenous ligands of the nuclear receptor liver X receptor α (LXRα; NR1H3) (8–10), which modulates immune responses and regulates various metabolic pathways, including cholesterol, bile acids, FAs, and glucose (11, 12).

In in vitro experiments, 25-hydroxycholesterol is widely used as a potent inhibitor of HMG-CoA reductase or as a ligand of LXRα, but the origins of this oxysterol are not entirely clear. Enzymatic production of 25-hydroxycholesterol has been reported by microsomal cholesterol 25-hydroxylase (CH25H) (13), and the activation of Toll-like receptors, a class of proteins that play a key role in the innate immune system, markedly induces CH25H and increases 25-hydroxycholesterol concentrations in mice macrophages and sera (14, 15). In comparison with mice, however, expression of CH25H has been reported to be very low in human tissues (13). Other enzymes involved in the production of 25-hydroxycholesterol are mitochondrial sterol 27-hydroxylase (CYP27A1) (16, 17) and brain-specific microsomal cholesterol 24*S*-hydroxylase (CYP46A1) (18). In addition, nonenzymatic generation of 25-hydroxycholesterol by autoxidation of cholesterol has also been described (19).

Previously, we measured hepatic concentrations of intermediates in bile acid synthesis in *Cyp27*$^{-/-}$ mice (20). In this series of analyses, we unexpectedly found that microsomal concentrations of 25-hydroxycholesterol were

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Abbreviations: CH25H, cholesterol 25-hydroxylase; CTX, cerebrotendinous xanthomatosis; CYP27A1, sterol 27-hydroxylase; CYP46A1, cholesterol 24*S*-hydroxylase; Insig, insulin-induced gene; LXR, liver X receptor; PCN, pregnenolone-16α-carbonitrile; SREBP, sterol-regulatory element binding protein; SRM, selected reaction monitoring.

[1]To whom correspondence should be addressed.
e-mail: ymatsuzaki-gi@umin.ac.jp

significantly elevated in *Cyp27*$^{-/-}$ mice (unpublished observation). This might be caused by reduced metabolism of 25-hydroxycholesterol due to inhibition of 27-hydroxylation. However, it was also possible that 25-hydroxylation of cholesterol was stimulated by enzyme upregulation in the *Cyp27*$^{-/-}$ mice. We speculated that CYP3A was the enzyme that exhibited high cholesterol 25-hydroxylation activity because CYP3A was markedly upregulated in *Cyp27*$^{-/-}$ mice and this enzyme was known to catalyze a similar reaction, i.e., 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol (21).

The CYP3A subfamily consists of monooxygenases that catalyze many reactions involved in the metabolism of xenobiotics, steroid hormones, and bile acids (22). Cholesterol is also one of the substrates for CYP3A and is believed to be mainly metabolized to 4β-hydroxycholesterol (23, 24). The present study was undertaken to prove that CYP3A catalyzes not only 4β-hydroxylation but also 25-hydroxylation of cholesterol and to show the possibility that 25-hydroxycholesterol in normal human serum originates from CYP3A4.

MATERIALS AND METHODS

Chemicals

Pregnenolone-16α-carbonitrile (PCN) and troleandomycin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Cholesterol and desmosterol were obtained from Steraloids, Inc. (Newport, RI), and cholesterol was used as substrate for the enzyme assay after purification with disposable silica cartridge columns (25) to remove contaminated oxysterols. Additional reagents and solvents were of analytical grade.

Cell culture

AML12 cells, a differentiated, nontransformed hepatocyte cell line that was derived from transforming growth factor α-overexpressing transgenic mice (26) were purchased from American Type Culture Collection (Manassas, VA). Cells were seeded in 6-well plates and cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (Invitrogen Japan KK; Tokyo, Japan) supplemented with 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 5 ng/ml selenium, 40 ng/ml dexamethasone, and 10% FBS. When the cells were subconfluent, the medium was replaced with fresh medium containing PCN, troleandomycin, or desmosterol dissolved in 1% ethanol. Although 1% ethanol in the medium had no detectable effects on cell growth, the same concentration of ethanol was also added to the control wells. Cells were incubated at 37°C in a humidified incubator containing 5% CO_2 and 95% air.

RNA measurements

Total RNA was extracted from the cells using an AllPrep RNA/protein kit (QIAGEN KK; Tokyo, Japan). Reverse transcription was performed on 1 μg of total RNA using a first-strand cDNA synthesis kit for RT-PCR (Roche Diagnostics; Mannheim, Germany). Real-time quantitative PCR was performed on cDNA aliquots with FastStart DNA Master SYBR Green I and a LightCycler (Roche). The sequences of the oligonucleotide primer pairs used to amplify mouse mRNAs are 5′-GGCAGCATTGATCCTTATG-3′ and 5′-AAGAACTCCTTGAGGGAGAC-3′ for *Cyp3a11* (NM_007818), 5′-ACACCTACTTTGAAGACCCAT-3′ and 5′-TGACAACTTTCACCTCCAT-3′ for *Cyp46a1* (NM_010010), 5′-CTTCCTGCTGACCAATGAAT-3′ and 5′-AGCTTTTAGCAGAGGCATGT-3′ for *Cyp27a1* (NM_024264), 5′-CCAGCTCCTAAGTCACGTC-3′ and 5′-CACGTCGAAGAAGGTCAG-3′ for *Ch25h* (NM_009890) and 5′-CCTGTATGCCTCTGGTCGTA-3′, and 5′-CCATCTCCTGCTCGAAGTCT-3′ for β-actin (X03672). PCR amplification began with a 10 min preincubation step at 95°C, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 62°C for 10 s, and elongation at 72°C for 16 s. The relative concentration of the PCR product derived from the target gene was calculated using LightCycler System software. A standard curve for each run was constructed by plotting the crossover point against the log concentration. The concentration of target molecules in each sample was then calculated automatically by reference to this curve ($r = -1.00$), and results were standardized to the expression of β-actin. The specificity of each PCR product was assessed by melting curve analysis.

SDS-PAGE and immunoblot analysis

Cell homogenate was resolved by SDS-PAGE on a 5–20% gradient gel (e-PAGEL; ATTO Corporation, Tokyo, Japan) and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA). Immunoblot analyses of mouse CYP3A, CH25H, and β-actin were conducted with goat polyclonal antibody against mouse CYP3A, goat polyclonal antibodies against human CH25H (Santa Cruz Biotechnology; Santa Cruz, CA), and mouse monoclonal anti-β-actin antibody (Sigma), respectively. The membrane was blocked for 1 h in 5% fat-free milk in TBS-T (Tris-buffered saline/0.1% Tween-20) and incubated with the primary antibody against either CYP3A (1:200 dilution), CH25H (1:200 dilution), or β-actin (1:1,000 dilution) in 5% fat-free milk in TBS-T overnight at 4°C. The blot was washed three times for 10 min in TBS-T and incubated with an HRP-conjugated donkey anti-goat IgG antibody (Santa Cruz Biotechnology) for CYP3A and CH25H or with an HRP-conjugated sheep anti-mouse IgG antibody (Amersham; Buckinghamshire, UK) for β-actin. After washing, the bands were visualized by exposure to film (Hyperfilm ECL; Amersham) with an ECL Western blotting analysis system (Amersham) according to the manufacturer's instructions. The gradient gel was calibrated with prestained molecular-weight markers (Bio-Rad Japan; Tokyo, Japan).

Sample collection from human subjects

Blood samples were collected from 78 healthy adults. After coagulation and centrifugation at 1,500 *g* for 10 min, serum samples were stored at −20°C until analysis. Informed consent was obtained from all subjects, and the experimental procedures were approved by the Teikyo University Institutional Review Board.

Determination of sterol concentrations

Sterol concentrations in cell homogenate and serum were measured using our previously described HPLC-ESI-MS/MS method (27, 28). In brief, 5 μl aliquots of serum or cell homogenate (approximately 1×10^4 cells) were incubated with stable isotope-labeled oxysterols as internal standards in 1 N ethanolic KOH at 37°C for 1 h. Sterols were extracted with *n*-hexane, derivatized to picolinyl esters, and analyzed by HPLC-ESI-MS/MS. Conventional derivatization was conducted at 80°C for 60 min, but room temperature for 30 min was chosen for the specific monopicolinyl ester formation of 25-hydroxycholesterol. Monopicolinyl 25-hydroxycholesterol exhibited $[M+Na+CH_3CN]^+$ ion as the base peak, and [picolinic acid $(C_6H_5NO_2)$+Na]$^+$ ion was observed as the most-abundant product ion under various levels of collision energy. Therefore, *m/z* 571 → 146 (25 V) and *m/z* 574 → 146 (25 V) were used as the monitoring ions and optimal

collision energy for authentic and deuterated 25-hydroxycholesterol monopicolinate, respectively. Essentially, the Hypersil GOLD column (150 mm × 2.1 mm ID, 3 μm; Thermo Fisher Scientific, San Jose, CA) was employed for the HPLC separation of sterols, and the Hypersil GOLD aQ column (150 mm × 2.1 mm ID, 3 μm) was also used to obtain better separation of the stereoisomers (29).

Enzyme assay

Microsomes (baculosomes) prepared from insect cells that were infected with a baculovirus containing the cDNA for rabbit cytochrome P450 reductase and human *CYP1A2*, *CYP2C9*, *CYP2D6*, or *CYP3A4* were purchased from Invitrogen. The microsomes (10 pmol of P450) were incubated for 30 min at 37°C with various amounts of cholesterol (dissolved in 12 μl of a 33% aqueous solution of 2-hydroxypropyl-β-cyclodextrin), NADPH (1.2 mM), glucose-6-phosphate (3.6 mM), 2 U glucose-6-phosphate dehydrogenase, and 100 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA in a total volume of 0.5 ml. The incubation was stopped by the addition of 1 ml ethanol. After the addition of the internal standards and 5 μg butylated hydroxytoluene to the mixture, oxysterols were extracted twice with 2 ml *n*-hexane, derivatized to picolinyl esters, and analyzed by HPLC-ESI-MS/MS, as described above. To exclude the possible effects of contaminated oxysterols in substrate (cholesterol) and cholesterol autoxidation, incubations without adding NADPH generating system were conducted simultaneously, as a control, and the data were subtracted from those obtained using complete assay mixtures. An assay using boiled CYP3A4 was also conducted to exclude the direct effects of the NADPH generating system on cholesterol oxidation.

Statistics

Data are expressed as the mean ± SD. The statistical significance of differences between the results in the different groups was evaluated using the Student's two-tailed *t*-test. Correlation was tested by calculating Pearson's correlation coefficient, *r*. For all analyses, significance was accepted at the level of $P < 0.05$.

RESULTS

The effects of PCN, troleandomycin, and desmosterol on sterol concentrations in AML12 cells are shown in **Fig. 1**. The concentrations of 4β-hydroxycholesterol, 25-hydroxycholesterol, and 22R-hydroxycholesterol were significantly increased by treatment with PCN, a classical inducer of CYP3A by the activation of pregnane X receptor (NR1I2) (22). In contrast, these oxysterol concentrations were significantly decreased by treatment with troleandomycin, a specific inhibitor of CYP3A activity (30). Furthermore, the increase of 25-hydroxycholesterol by PCN treatment was not suppressed by the addition of desmosterol, a potent inhibitor of CH25H (13). On the other hand, significant increase by PCN was not observed regarding the other oxysterol concentrations.

The effects of PCN, troleandomycin, and desmosterol on mRNA expressions of *Cyp3a11*, *Ch25h*, *Cyp46a1*, and *Cyp27a1* in AML12 cells are shown in **Fig. 2**. Treatment with PCN significantly upregulated *Cyp3a11* expression. Marked upregulation of *Ch25h* expression was also observed in the PCN-treated cells, but the absolute mRNA expression of *Ch25h* in untreated AML12 cells was more than 50 times lower than that of *Cyp3a11* (data not shown). Troleandomycin tended to upregulate the mRNA expression of *Cyp3a11*, but the difference was not statistically significant. The addition of desmosterol to cell culture medium did not affect the induction of *Cyp3a11* by PCN. However, desmosterol seemed to inhibit the induction of *Ch25h* by PCN.

Figure 3 shows the effects of PCN, troleandomycin, and desmosterol on protein levels of CYP3A and CH25H. PCN increased CYP3A protein level, which was associated with the upregulated transcription of *Cyp3a11* (Fig. 2). However, although the transcription of *Ch25h* was also upregulated by the addition of PCN, the protein level of CH25H was not elevated. In addition, desmosterol did not affect the expression of cellular CYP3A protein, but CH25H protein level was obviously decreased by desmosterol treatment.

Intact or boiled aliquots of insect cell microsomes overexpressing recombinant human CYP3A4 (10 pmol of P450) were incubated at 37°C for 30 min with 200 μM cholesterol and an NADPH generating system, and the sterol fraction was derivatized to picolinyl esters by two different methods. **Figures 4A, C** represent selected reaction monitoring (SRM) of samples that were derivatized at 80°C for 60 min. This derivatizing method generally produced di-picolinyl esters of oxysterols, and the SRM data indicated that 25-hydroxycholesterol was a major product of intact CYP3A4, as well as 4β-hydroxycholesterol. We also derivatized the sample at room temperature for 30 min, which produced mono-picolinyl ester of 25-hydroxycholesterol (Fig. 4B, D). The mass spectrum and retention time of mono-picolinyl 25-hydroxycholesterol are completely distinct from those of di-picolinyl 25-hydroxycholesterol. The production of 25-hydroxycholesterol by intact CYP3A4 was confirmed using this specific derivatization technique.

The effects of substrate (cholesterol) concentrations on various hydroxylase activities in recombinant human CYP3A4 are presented in **Fig. 5**. The most significant activity was 25-hydroxylation, which was higher than that of 4β-hydroxylation, a marker activity of CYP3A4. Other hydroxylation activities, i.e., 22R-, 24R-, 24*S*-, 26-, and 27-hydroxylation were also observed, but the activities were much lower than that of 4β-hydroxylation. Apparent V_{max} and K_m were calculated by Lineweaver-Burk plots. V_{max} of 25-, 4β-, 22R-, 24R-, 24*S*-, 26-, and 27-hydroxylation were 7.0×10^{-4}, 2.0×10^{-4}, 5.7×10^{-5}, 5.8×10^{-5}, 3.4×10^{-6}, 5.3×10^{-5}, and 2.3×10^{-5} mol/s/mol P450, respectively, and K_m of those hydroxylations were 182, 62, 37, 161, 15, 80, and 45 μM, respectively.

In **Table 1**, cholesterol 25- and 4β-hydroxylase activities are compared among four different insect cell microsomes containing recombinant human CYP1A2, CYP2C9, CYP2D6, or CYP3A4. Not only CYP3A4 but also the other three P450 enzymes significantly catalyzed 25-hydroxylation of cholesterol, but these activities were lower than that by CYP3A4. In contrast, 4β-hydroxylation of cholesterol was exclusively observed in microsomes containing CYP3A4. Control microsomes without expressed human P450 enzymes did not convert cholesterol into 25-hydroxycholesterol or 4β-hydroxycholesterol.

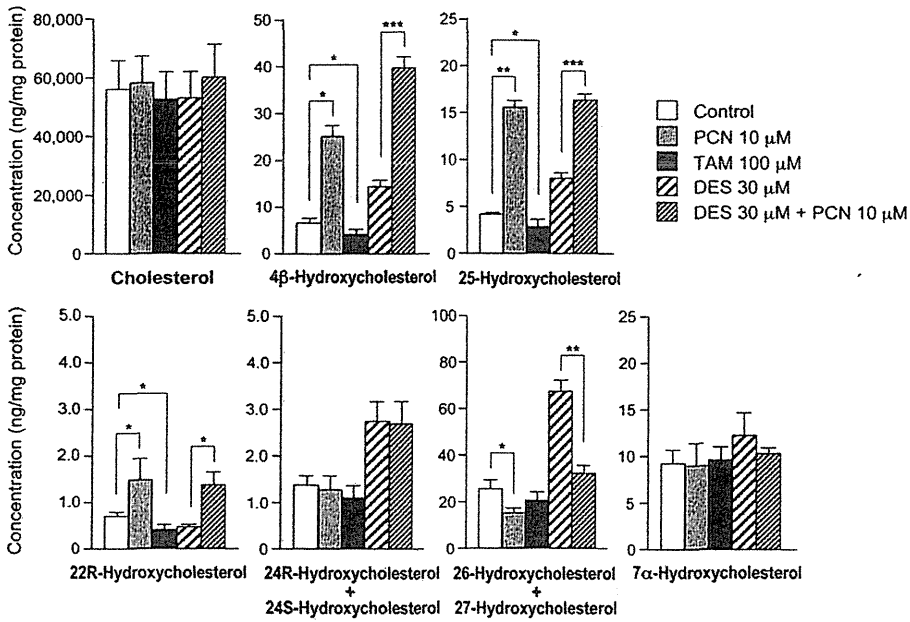

Fig. 1. Effects of PCN, troleandomycin (TAM), and desmosterol (DES) on sterol concentrations in AML12 cells. Cells were incubated with PCN (10 μM), TAM (100 μM), DES (30 μM), or DES (30 μM) plus PCN (10 μM) for 7 days. A Hypersil GOLD column was used for HPLC separation of oxysterols. This column cannot distinguish between 26- and 27-hydroxycholesterol and between 24R- and 24*S*-hydroxycholesterol. Each column and error bar represents the mean and SD obtained in triplicate assay. $***P < 0.001$, $**P < 0.01$, $*P < 0.05$.

The relationships between serum 25-hydroxycholesterol concentrations and serum 4β-, 24*S*-, and 27-hydroxycholesterol concentrations in 78 normal Japanese subjects are shown in **Fig. 6**. Serum 25-hydroxycholesterol concentrations correlated significantly with 4β-hydroxycholesterol concentrations (Fig. 6A), but did not correlate significantly with the concentrations of 24*S*-hydroxycholesterol (Fig. 6B) or 27-hydroxycholesterol (Fig. 6C). On the other hand, serum 24*S*-hydroxycholesterol and 27-hydroxycholesterol concentrations correlated significantly ($r = 0.408$, $P < 0.0005$, n = 78) in the group of normal subjects.

DISCUSSION

Our results provide strong evidence that 25-hydroxylation of cholesterol is catalyzed by CYP3A. First, CYP3A induction caused the accumulation of 25-hydroxycholesterol in a cell line derived from mouse liver. The addition of desmosterol downregulated CH25H protein in the cells, but did not reduce the concentration of cellular 25-hydroxycholesterol. Second, the presence of significant cholesterol 25-hydroxylation activity was proven by using recombinant human CYP3A4. Third, 25-hydroxycholesterol concentrations in normal human sera correlated positively with the 4β-hydroxycholesterol level; a known marker of CYP3A4 activity (23, 24).

In this study, we paid close attention to identifying 25-hydroxycholesterol by using two different derivatization methods, i.e., 80°C for 60 min and room temperature for 30 min. The former method synthesizes the usual dipicolinyl derivative of 25-hydroxycholesterol, whereas the latter method produces the mono-picolinyl derivative, because the C-25 position of 25-hydroxycholesterol is resistant to picolinyl ester formation at room temperature (28). The identification of 25-hydroxycholesterol by our conventional HPLC-MS/MS method was confirmed using

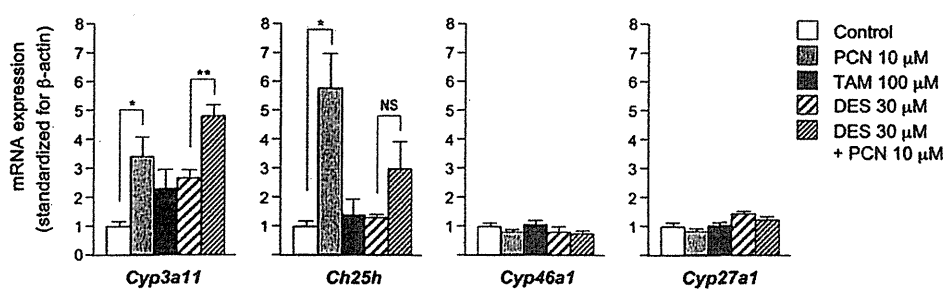

Fig. 2. Effects of PCN, troleandomycin (TAM), and desmosterol (DES) on relative mRNA expression of *Cyp3a11*, *Ch25h*, *Cyp46a1*, and *Cyp27a1* in AML12 cells. Cells were incubated with PCN (10 μM), TAM (100 μM), DES (30 μM), or DES (30 μM) plus PCN (10 μM) for 72 h. Each column and error bar represents the mean and SD obtained in triplicate assay. $**P < 0.01$, $*P < 0.05$. NS, not significant.

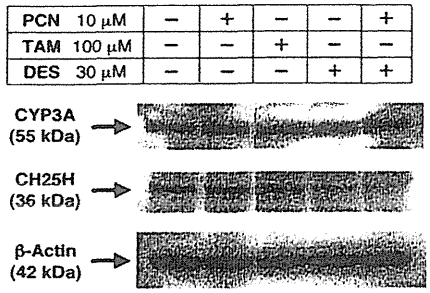

Fig. 3. Effects of PCN, troleandomycin (TAM), and desmosterol (DES) on CYP3A and CH25H protein in AML12 cells. Cells were incubated with PCN (10 μM), TAM (100 μM), DES (30 μM), or DES (30 μM) plus PCN (10 μM) for 72 h. Cell homogenates (10 μg protein per lane) were subjected to SDS-PAGE analysis.

this specific derivatization technique. Furthermore, we quantified 25-hydroxycholesterol with great care because this oxysterol may be a normal contaminant of the substrate (cholesterol) and could be generated by cholesterol autoxidation. Therefore, in the recombinant cytochrome P450 experiments, control assays without adding the NADPH generating system were conducted simultaneously and the data were subtracted from those obtained using the complete assay system.

It was surprising that recombinant CYP3A4 produced much more 25-hydroxycholesterol than 4β-hydroxycholesterol, which is used as a marker of CYP3A4 activity (23, 24). However, serum concentrations of 25-hydroxycholesterol were low compared with those of 4β-hydroxycholesterol (Fig. 6A), which may be explained by the fact that the metabolism of 25-hydroxycholesterol is faster than that of 4β-hydroxycholesterol (31). Whereas 4β-hydroxycholesterol is metabolized slowly by CYP7A1 and CYP27A1 (31), 25-hydroxycholesterol is metabolized faster by CYP7A1 (32) and CYP7B1 (33).

It has been reported that 25-hydroxycholesterol is synthesized not only by CH25H (13) but also by CYP27A1 (16, 17) and CYP46A1 (18). Because only very low levels of

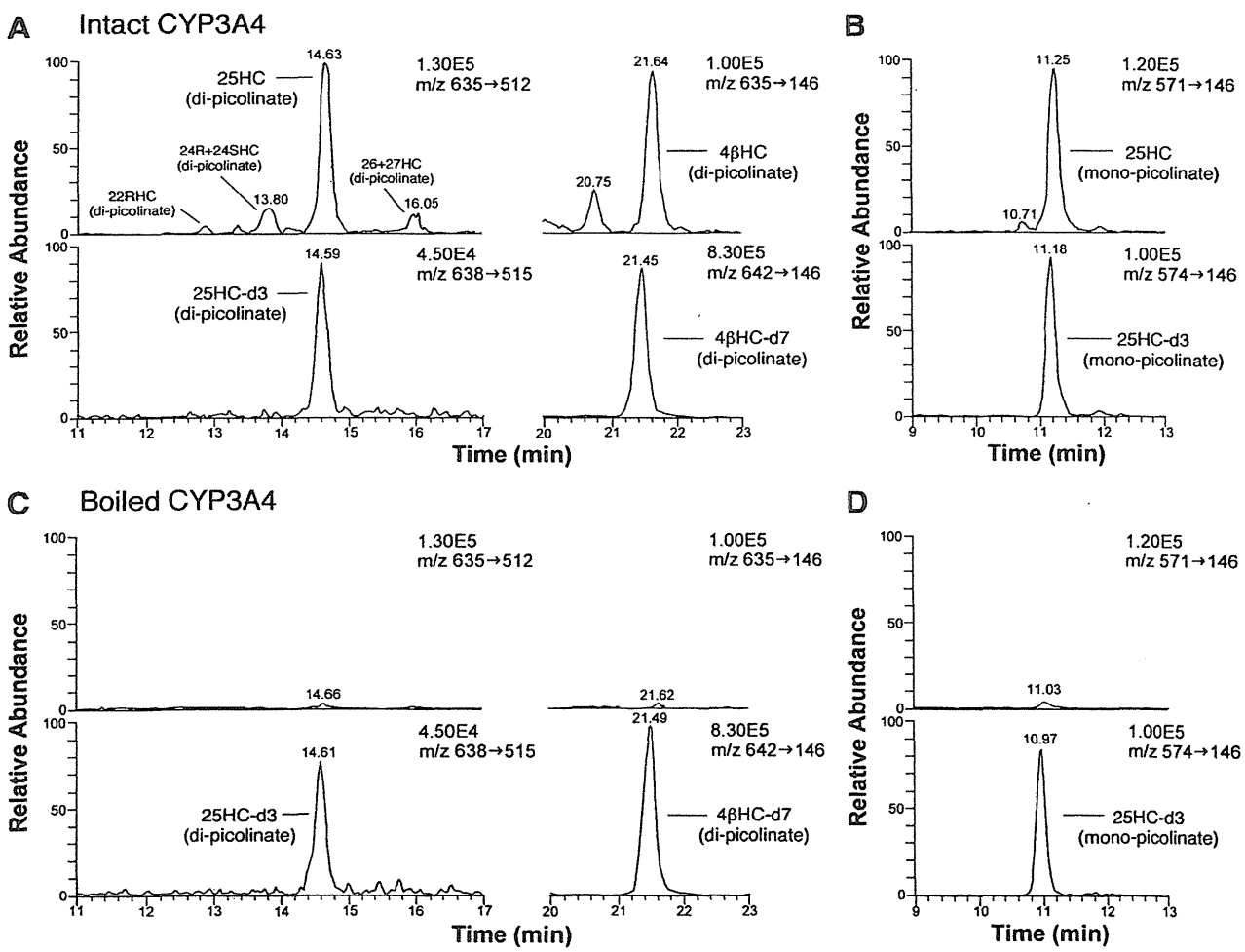

Fig. 4. SRM chromatograms obtained during HPLC-ESI-MS/MS analysis of the oxysterol fraction from an incubation mixture of overexpressed recombinant human CYP3A4 (A, B) or boiled CYP3A4 (C, D) with 200 μM cholesterol. The oxysterol fraction was derivatized to picolinyl esters by two different methods, 80°C for 60 min (A, C) and room temperature for 30 min (B, D). The former produces di-picolinyl esters of 25-hydroxycholesterol (25HC) and 4β-hydroxycholesterol (4βHC), whereas the latter produces the mono-picolinyl ester of 25HC. 25HC-d3 (1 ng) and 4βHC-d7 (5 ng) were added to each incubated mixture as internal standards. The same Hypersil GOLD column and the same mobile phase were used for HPLC separation of both di- and mono-picolinyl esters of 25HC. The numbers on the right upper side of each chromatogram represent the full scale of the chromatogram.

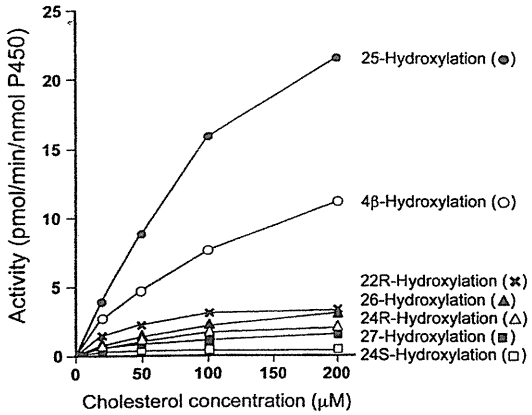

Fig. 5. Effects of cholesterol (substrate) concentrations on 25-, 4β-, 22R-, 24R-, 24*S*-, 26-, and 27-hydroxylase activities in overexpressed recombinant human CYP3A4. A Hypersil GOLD aQ column was used for HPLC separation of oxysterols. Data points represent the mean of duplicate determinations.

CH25H are expressed in normal human tissues (13), the roles of CYP27A1 and CYP46A1 in the formation of 25-hydroxycholesterol may be relatively important in humans. However, our results showed that the serum concentrations of 25-hydroxycholesterol did not correlate with the concentrations of either 27-hydroxycholesterol, a product of CYP27A1, or 24*S*-hydroxycholesterol, a product of CYP46A1. In contrast, 25-hydroxycholesterol levels were significantly correlated with 4β-hydroxycholesterol concentrations in normal human subjects. The results lend support to the hypothesis that CYP3A4 synthesizes 25-hydroxycholesterol, as well as 4β-hydroxycholesterol.

Our results showed that not only CYP3A4 but also CYP1A2, CYP2C9, and CYP2D6 catalyzed 25-hydroxylation of cholesterol to some extent (Table 1). However, CYP3A4 is the most abundantly expressed form of P450 in human liver (as much as 60% of all hepatic P450) (34). In addition, because cholesterol 4β-hydroxylase activities by CYP1A2, CYP2C9, and CYP2D6 were negligible, the positive correlation between serum concentrations of 25-hydroxycholesterol and 4β-hydroxycholesterol cannot be explained by these P450 activities. Thus, at least in normal human subjects, most of the serum 25-hydroxycholesterol appears to originate from CYP3A4.

Under abnormal conditions, however, serum 25-hydroxycholesterol concentrations may not change with 4β-hydroxycholesterol levels. For example, in a patient with cerebrotendinous xanthomatosis (CTX), *CYP27A1* deficiency, serum 25-hydroxycholesterol concentration was low but 4β-hydroxycholesterol concentration was high compared with those in a normal subject (28). Because CYP3A4 activity is not significantly altered in CTX (21), it is likely that these oxysterol concentrations were affected by the activities of other enzymes, i.e., impaired CYP27A1 and upregulated CYP7A1 (21) that metabolize 4β-hydroxycholesterol and 25-hydroxycholesterol, respectively. A recent report by Diczfalusy et al. (15) showed that intravenous injection of lipopolysaccharide (endotoxin) in healthy volunteers resulted in an increase in plasma 25-hydroxycholesterol concentration. Although CH25H activity was not determined in these subjects, the increase might be due to the induction of CH25H, as suggested by their experiments using mouse macrophage.

The biochemical role of the production of 25-hydroxycholesterol by CYP3A remains unclear. However, this oxysterol appears to be further metabolized to bile acids (35), which may be one of the important alternative pathways for bile acid biosynthesis. In addition, this oxysterol is a potent inhibitor of HMG-CoA reductase and a ligand of LXRα, so that it may participate in the regulation of lipid metabolism. It should be noted that CYP3A4 catalyzes not only 25-hydroxylation but also 4β-hydroxylation, 22R-hydroxylation, and other nonstereospecific hydroxylations of cholesterol, including 24R-, 24*S*-, 26-, and 27-hydroxylation (Fig. 5). Because 4β-hydroxycholesterol, 22R-hydroxycholesterol, and 24*S*-hydroxycholesterol have been reported to be more potent activators of LXRα compared with 25-hydroxycholesterol (8, 9), the influence of CYP3A induction on LXRα activity is not explained by the effects of 25-hydroxycholesterol alone.

Fatty liver and hypertriglyceridemia are characteristic features in *Cyp27*$^{-/-}$ mice (36) but not in CTX patients. Because CYP3A is markedly upregulated in *Cyp27*$^{-/-}$ mice but not in CTX patients (21), oxysterols synthesized by CYP3A may induce fatty liver in *Cyp27*$^{-/-}$ mice. In fact, SREBP1, a target gene of LXRα, and SREBP1-regulated FA biosynthetic enzymes were upregulated in *Cyp27*$^{-/-}$ mice (36), whereas SREBP1 was not upregulated in CTX patients (37).

TABLE 1. Cholesterol 25- and 4β-hydroxylation activities in recombinant overexpressed human cytochrome P450 (baculosomes[a])

	P450 concentration	25-Hydroxylation[b]		4β-Hydroxylation[b]	
Baculosomes	*pmol P450/mg protein*	*pmol/min/mg protein*	*pmol/min/nmol P450*	*pmol/min/mg protein*	*pmol/min/nmol P450*
WT control[c]	0	0.06 (0.07, 0.05)	—	0.01 (0.00, 0.01)	—
CYP1A2	98	0.58 (0.54. 0.62)	5.95 (5.54, 6.35)	0.12 (0.13, 0.11)	1.21 (1.30, 1.12)
CYP2C9	313	1.36 (1.50, 1.21)	4.34 (4.79, 3.89)	0.25 (0.28, 0.21)	0.79 (0.90, 0.68)
CYP2D6	252	0.59 (0.64, 0.54)	2.36 (2.56, 2.15)	0.14 (0.17, 0.10)	0.54 (0.69, 0.39)
CYP3A4	96	1.86 (2.07, 1.64)	19.4 (21.6, 17.1)	0.99 (1.08, 0.89)	10.3 (11.2, 9.31)

WT, wild type.

[a]Microsomes prepared from insect cells that were infected with baculovirus containing the cDNAs for human cytochrome P450 and rabbit cytochrome P450 reductase.

[b]Average of two assays. Individual values in parentheses.

[c]Control microsomes prepared from insect cells that were infected with a wild-type baculovirus.

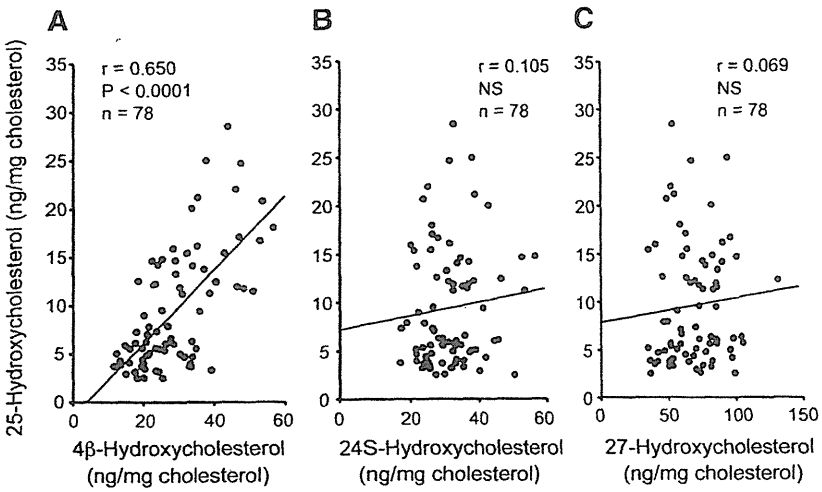

Fig. 6. Correlations between serum concentrations of 25-hydroxycholesterol and 4β-hydroxycholesterol (A), 24S-hydroxycholesterol (B), or 27-hydroxycholesterol (C) in 78 normal subjects. NS, not significant.

In summary, 25-hydroxycholesterol was quantified using the latest HPLC-ESI-MS/MS technique in a mouse liver cell line, in microsomes overexpressing recombinant human cytochrome P450 enzymes and in normal human sera. All data support the idea that CYP3A was one of the responsible enzymes that catalyzed the 25-hydroxylation of cholesterol.

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Review Article

Cholesterol and chronic hepatitis C virus infection

Akira Honda and Yasushi Matsuzaki

Department of Gastroenterology, Tokyo Medical University Ibaraki Medical Center, Ibaraki, Japan

Cholesterol is an essential molecule for the life cycle of the hepatitis C virus (HCV). This review focuses on the roles of cholesterol in HCV infection and introduces HCV events related to cholesterol metabolism and applications for cholesterol metabolism as a therapeutic target. HCV appears to alter host lipid metabolism into its preferable state, which is clinically recognized as steatosis and hypocholesterolemia. While hepatic fatty acid and triglyceride syntheses are upregulated in chronic hepatitis C patients, no direct evidence of increased hepatic *de novo* cholesterol biosynthesis has been obtained. Impaired VLDL secretion from hepatocytes is suggested to increase intracellular cholesterol concentrations, which may lead to hypocholesterolemia. Clinically, lower serum cholesterol levels are associated with lower rates of sustained virological responses (SVR) to pegylated-interferon plus ribavirin therapy, but the reason remains unclear. Clinical trials targeting HMG-CoA reductase, the rate-limiting enzyme in the cholesterol biosynthetic pathway, are being conducted using statins. Anti-HCV actions by statins appear to be caused by the inhibition of geranylgeranyl pyrophosphate synthesis rather than their cholesterol lowering effects. Other compounds that block various steps of cholesterol metabolic pathways have also been studied to develop new strategies for the complete eradication of this virus.

Key words: cholesterol, hepatitis C, HMG-CoA reductase, hypocholesterolemia, statins, steatosis

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection is deeply associated with lipid disorders because this virus utilizes host lipid metabolism to sustain its life cycle.[1–3] Accordingly, understanding lipid metabolism in HCV infection is necessary to develop new strategies for the complete eradication of this virus. Characteristic lipid disorders observed in chronic hepatitis C patients are steatosis and hypocholesterolemia,[4] which are mainly caused by the abnormal metabolism of triglyceride and cholesterol, respectively. The metabolic pathways of these two lipids are closely related but are virtually different from each other. In this review, we focus on the roles of cholesterol in persistent HCV infection and introduce recent developments in studies concerning HCV-altered events of cholesterol metabolism and the application towards therapies targeting the cholesterol metabolic pathways.

Correspondence: Dr Yasushi Matsuzaki, Department of Gastroenterology, Tokyo Medical University Ibaraki Medical Center, 3-20-1 Chuoh, Ami, Inashiki, Ibaraki 300-0395, Japan. Email: ymatsuzaki-gi@umin.ac.jp

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ROLES OF CHOLESTEROL IN THE LIFE CYCLE OF HCV

Cholesterol as a component of HCV

CHOLESTEROL IS AN important molecule for eukaryotic cell membranes and many eukaryotes possess the ability to synthesize cholesterol. In contrast, the ability of cholesterol synthesis is rarely found in prokaryotes, and the lack of cholesterol in cell membranes is frequently cited as a characteristic feature of prokaryotes.[5] Viruses do not have a cellular structure or their own metabolism. However, some contain high levels of cholesterol, which represents 38–49 mol% of the total virus lipid and more than 1% of the total mass of the virion.[6,7] The lipid is generally present in a limiting membrane structure termed the viral envelope, which is derived from a portion of the host eukaryotic cell membranes. This viral envelope surrounds the viral nucleocapsid assembly, and viruses with this structure are referred to as enveloped viruses.

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HCV is one of the enveloped viruses belonging to the *Flaviviridae* family. Because of the lack of an efficient cell culture system or a suitable animal model, the detailed life cycle of HCV was unclear for a long time. However, in 1999 Lohmann *et al.*[8] reported subgenomic HCV replicons that self-amplify in cultured human hepatoma cells, Huh-7. Moreover, the generation of infectious HCV upon transfection of these cells with a particular molecular HCV clone called Japanese fulminant hepatitis-1 (JFH1, genotype 2a) was established in 2005.[9–11] Using this system, it was ascertained that mature HCV virions were also enriched with cholesterol and cholesterol depletion from the virus resulted in a complete loss of infectivity.[12] In addition, the cholesterol/phospholipid molar ratio was significantly higher in JFH1 viruses (1.29) compared with that in the host Huh-7 cell membranes (0.40 and 0.42 for JFH1-infected and uninfected cells, respectively).[12]

The cholesterol/phospholipid molar ratio is one of the parameters related to membrane viscosity.[13] A decrease in the ratio markedly increases the fluidity of the membrane, which might induce a conformational change of the viral envelope, resulting in the loss of infectivity.

The higher cholesterol/phospholipid ratio in HCV virions compared with the host cell membranes suggests that the viral envelope is not derived from usual plasma or endoplasmic reticulum (ER) membrane but from a special membrane that is rich in cholesterol. A recent study demonstrated that HCV induced the apposition of lipid droplets to ER-derived membranes and that HCV assembly took place within this lipid droplet-associated membrane.[14] It may be possible that the concentration of cholesterol in this particular membrane is exceptionally high compared with the other membranes in the host cells.

Association with lipoproteins

Cholesterol is one of the central components of lipoproteins. The physiological function of the lipoproteins is to transport and deliver lipids (mainly triglyceride and cholesterol) throughout the body in the blood. HCV utilizes this transportation system to establish its own life cycle.[3] The viruses interact with human lipoproteins of various densities and form lipo-viral particles (LVPs).[15–17] However, the lower density particles, very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL), are more effective at transmitting infection to chimpanzees and cultured hepatocytes.[18,19] Specific amino acid sequences in HCV envelope proteins (E1 and E2) play critical roles in binding HCV particles to the lipoproteins.[20]

Apolipoprotein B-100 (ApoB-100) is found in LDL and VLDL particles and there is only one ApoB-100 per particle.[21] Therefore, the number of these particles in serum is calculated by using the serum concentration (ca 80 mg/dL) and molecular weight (513 000) of ApoB-100, for a concentration of 1.56×10^{-6} mol/L. On the other hand, serum HCV core antigen levels in chronic hepatitis C patients can reach 6×10^{-11} mol/L.[22,23] Thus, in human serum there are a sufficient number of lower density lipoprotein particles to compose LVPs with HCV virion. The diameter of VLDL is similar to that of HCV virion (55-65 nm),[24] while LDL and high-density lipoprotein (HDL) are much smaller than HCV. Therefore, as demonstrated in a recent study,[25] the average diameter of LVPs in patient serum does not exceed that of the HCV virion.

The formation of LVPs (as infectious HCV particles) appears to occur during lipoprotein synthesis in HCV infected hepatocytes rather than while circulating in the patient blood. The most abundant lipoprotein synthesized in the liver is VLDL, which consists of a central core that is rich in neutral lipids, triglyceride and cholesterol, surrounded by a phospholipid monolayer with ApoB-100 and apolipoprotein E (ApoE).[26] VLDL carries newly synthesized triglyceride from the liver to adipose tissues, and then delivers the resulting cholesterol to peripheral tissues as LDL. The first step of VLDL assembly in hepatocytes is the co-translational lipidation of ApoB-100 by microsomal triglyceride transfer protein (MTP).[27]

This partially lipidated pre-VLDL particle then matures into VLDL by fusing with ApoE and a large lipid droplet containing cholesterol ester and additional triglyceride.[28] The importance of VLDL synthesis in the secretion of LVPs is suggested by the fact that inhibition of ApoB-100,[29,30] ApoE[31,32] or MTP activity[29,30,33] suppresses the release of infectious HCV particles. In regard to ApoE, Hishiki *et al.* reported recently that HCV infectivity was influenced by ApoE isoforms.[34] LVPs containing ApoE2 isoform, which has low affinity for the LDL receptor (LDLR), were not as infectious as those with other isoforms, ApoE3 and ApoE4.

The mechanisms of cell entry by HCV are still incompletely understood.[35–37] However, recent data show that it is a complex multi-step process involving the presence of several entry factors. The HCV LVPs use the cell surface LDLR,[18] scavenger receptor class B type I (SR-BI)[38] and glycosaminoglycans, such as heparan sulphate[39] as initial attachment factors. LDLR and SR-BI

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recognize LDL and HDL of LVPs, respectively, and these receptors also interact with VLDL.[19,40] In contrast, heparan sulphate interacts directly with envelope glycoprotein E2 of HCV.[39] The virus that has attached to hepatocyte subsequently interacts with tetraspanin CD81[41] to form a complex with SR-BI, and is transferred to the tight junction proteins claudin-1, 6 and 9[42] and occludin.[43,44] Thus, cellular proteins that normally assemble to form firm seals between adjacent cells appear to provide the final entry key for HCV.[45]

HYPOCHOLESTEROLEMIA IN CHRONIC HCV INFECTION

ALTHOUGH SERUM LIPOPROTEINS appear to be essential for the life cycle of HCV, paradoxical hypocholesterolemia has frequently been described in patients with chronic hepatitis C.[46–58]

Through these reports the following results have been obtained: (i) Hypocholesterolemia is a well-known serological feature of patients with HCV genotype 3,[48–50,52] but other genotypes also cause significant hypocholesterolemia compared with normal subjects;[51,53,57,58] (ii) Hypocholesterolemia is caused mainly by the decrease of LDL cholesterol (β-lipoprotein),[53,55–57] but reduction of HDL cholesterol has also been reported;[52,58] (iii) In many cases in addition serum cholesterol, triglyceride concentrations are also reduced in patients,[53–56,58] whereas triglyceride levels are not decreased in some reports[52,57] likely due to data from obese subjects (BMI >25); (iv) The hypolipidemia is reversible after successful eradication of HCV but persists in nonresponders.[48–50,53,57,59]

It remains controversial whether the decrease of serum lipids is a direct effect of HCV infection or the result of hepatic damage caused by hepatitis.[60,61] The liver plays an important role in lipid metabolism and it may be influenced by hepatic inflammation. However, the hypolipidemia observed in HCV infection appears to be due to the modulation of lipid metabolism by HCV because patients with chronic hepatitis C have significantly lower cholesterol concentrations than those with chronic hepatitis B infection.[46–48,51] In addition, 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) that were not obese (BMI <25) (Fig. 1).[58]

The mechanisms responsible for the hypocholesterolemia in patients with HCV infection have not been elucidated. However, the following results lend support to the idea that impaired VLDL secretion from hepatocytes contributes to the reduction in serum cholesterol concentration: (i) Reduced cholesterol concentrations are usually associated with low triglyceride levels.[53–56,58] VLDL is known as a carrier for triglyceride, but also contains significant amount of cholesterol. After carrying triglyceride to adipose tissues, the resulting cholesterol is delivered to peripheral tissues as LDL; (ii) The degree of steatosis is inversely related with the serum concentration of cholesterol,[48] triglyceride[48] and ApoB[48,50] in non-obese (BMI <25), chronic hepatitis C patients; (iii) Inhibition of VLDL secretion by MTP inhibitors[62,63] or mipomersen,[64,65] an antisense oligonucleotide to reduce hepatic ApoB-100 synthesis, significantly lowers serum triglyceride and LDL cholesterol concentrations and elevates hepatic fat content; (iv) The expression levels of LDLR mRNA are markedly downregulated in livers of patients who are infected with HCV.[66,67] Serum LDL concentrations are controlled by the input and output of this lipoprotein. If output (LDLR activity) is inhibited, only a reduction of input (VLDL secretion from hepatocytes etc.) can explain the reduced serum level of LDL.

SERUM CHOLESTEROL LEVELS AND TREATMENT EFFICACY

SEVERAL STUDIES HAVE indicated that higher concentrations of serum cholesterol and LDL before treatment are important predictors of higher rates of sustained virological responses (SVR).[68–73] Through data mining analyses, Kurosaki *et al.* recently built models for pretreatment prediction of rapid virological response (RVR), complete early virological response (cEVR) or SVR to pegylated-interferon (PEG-IFN) plus ribavirin therapy for genotype 1 chronic hepatitis C.[74–76] Their original decision tree model included simple host factors, such as hepatic steatosis, serum LDL cholesterol, age, blood sugar and γ-glutamyltransferase (GGT).[74] The variable of initial split is the grade of steatosis. Among patients with hepatic steatosis of less than 30%, subjects with higher LDL cholesterol levels (≥100 mg/dL) are more likely to achieve RVR/cEVR (57% vs. 32%).

Another decision tree model includes viral factors, such as the interferon sensitivity determining region (ISDR) and the amino acid sequence of the HCV core protein (Core 70), as well as host factors, such as age, serum LDL cholesterol and hepatic fibrosis.[75] The ISDR is a stretch of 40 amino acids in the nonstructural 5A (NS5A) region of HCV and substitutions in ISDR are

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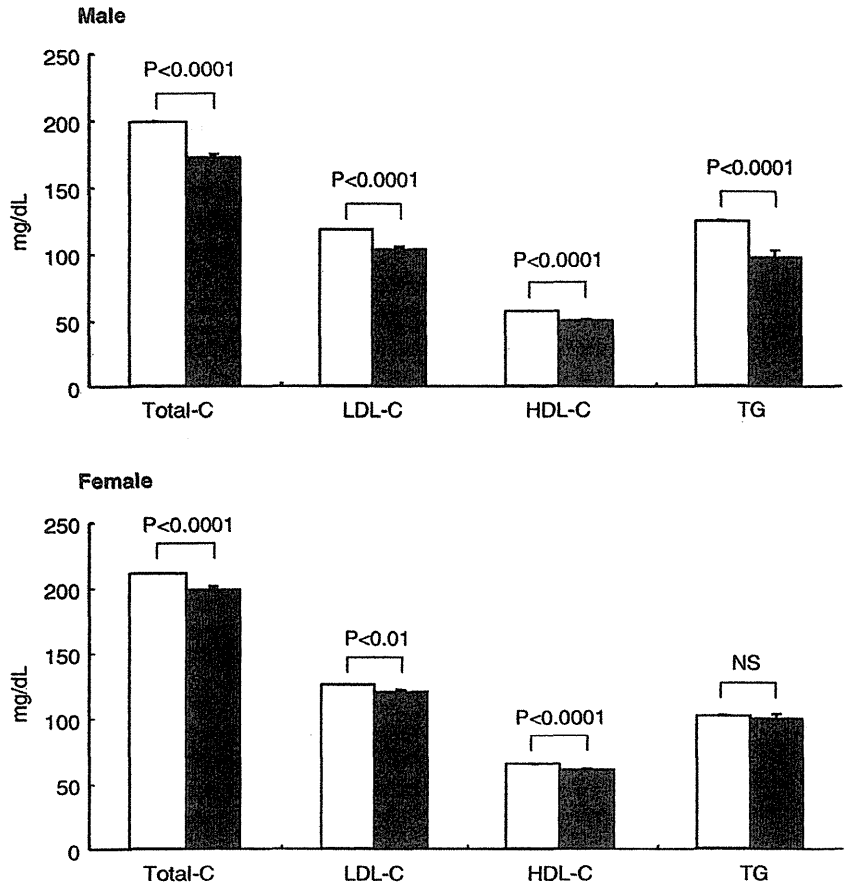

Figure 1 Comparison of serum total cholesterol (Total-C), LDL cholesterol (LDL-C), HDL cholesterol (HDL-C) and triglyceride (TG) concentrations between HCV-negative and -positive subjects with normal serum aminotransferase levels (ALT ≤30 and AST ≤30) without obesity (BMI <25). Data are expressed as the mean ± SD. Original data were reported previously by Miyazaki *et al.*[58] (Male: □ HCV-negative (N = 25 323), ■ HCV-positive (N = 118); Female: □ HCV-negative (N = 64 706), ■ HCV-positive (N = 210))

closely associated with SVR.[77–82] In contrast, amino acid substitutions at positions 70 and 91 of the core region have been reported to be associated with non-virological response to therapy.[70,83,84] The overall rate of SVR was only 44% in patients with a low number of substitutions in ISDR (0-1) but was increased 83% in selected subgroups of younger patients (<60 years) with the wild-type sequence at Core amino acid 70, and higher concentrations of LDL cholesterol (≥120 mg/dL).[75]

Recent studies have shown that single nucleotide polymorphisms located in the gene region encoding interleukin 28B (*IL28B*, also called *IFNλ3*) are strongly associated with the response to PEG-IFN plus ribavirin therapy.[85–87] The latest decision tree model, starting with one of the *IL28B* polymorphisms (rs8099917), includes platelet counts, ISDR and serum HCV-RNA levels, but does not include serum LDL cholesterol concentrations.[76] However, the total cholesterol, LDL cholesterol and ApoB concentrations are significantly higher in chronic hepatitis C patients carrying another *IL28B* major (responder) allele (CC in rs12979860) compared with those with minor (non-responder) alleles (CT or TT).[88] Therefore, the association between serum LDL cholesterol concentration and SVR may be reflected in this decision tree model by the underlying link between *IL28B* genotypes and LDL cholesterol concentrations.

It is not clear why a higher probability of SVR is predicted in patients with high serum cholesterol concentrations. As mentioned above, we cannot exclude the possibility that the high cholesterol levels in patients with HCV only reflect having the *IL28B* major (responder) allele. Otherwise, it may just reflect the wild-type sequence at Core amino acid 70 because substitution in the core protein correlated significantly with low concentration of LDL cholesterol.[89] However, in chronic hepatitis C patients, serum LDL cholesterol concentrations correlate negatively with hepatic LDLR mRNA expression[67] and monocyte LDLR protein expression.[90] These results indicate that high serum cholesterol

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concentrations are associated with downregulated LDLR, one of the putative receptors mediating HCV cell entry. In addition, adequate amounts of natural LDLR ligands, LDL and VLDL, might inhibit lipoprotein-mediated HCV cell entry via LDLR.[91]

ABNORMAL CHOLESTEROL METABOLISM IN HEPATOCYTES

STEATOSIS IS FREQUENTLY observed in HCV infection.[92] A comprehensive analysis of HCV core gene transgenic mice has shown that steatosis is mediated in large part by the HCV core protein.[93,94] At least four mechanisms have been suggested regarding the development of steatosis via the HCV core protein: (i) Inhibited tyrosine phosphorylation of insulin receptor substrate (IRS)-1 causes insulin resistance, which increases the peripheral release and hepatic uptake of fatty acids;[95] (ii) The suppression of MTP activity inhibits the secretion of VLDL from the liver;[96] (iii) Upregulated sterol regulatory element-binding protein (SREBP)-1c via the liver X receptor (LXR)-α pathway stimulates fatty acid synthesis in the liver.[97] In addition, the interaction between nuclear proteasome activator PA28γ and HCV core protein plays a critical role in the activation of this LXRα pathway; (iv) Downregulated peroxisome proliferator-activated receptor (PPAR)-α inhibits β-oxidation of fatty acids.[98,99] On the other hand, Huh-7 cells transfected with NS2[100] or 4B (NS4B)[101] from HCV have shown that these nonstructural proteins also upregulate SREBP-1c and appear to contribute to HCV-associated steatosis.

Steatosis is defined as an accumulation of lipid droplets, consisting mainly of triglycerides. The above mechanisms of steatosis may explain triglyceride accumulation in livers infected with HCV. However, the effects of HCV infection on hepatic cholesterol metabolism are poorly understood. Significant amounts of cholesterol are likely to be included within the lipid droplets, but data regarding the quantity of cholesterol in livers infected with HCV are limited. Woodhouse *et al.* reported for the first time that cholesterol concentrations in HCV-infected human hepatoma cell line Huh-7.5 were increased 56% compared with those in non-infected Huh-7.5 cells.[102] To understand the changes in cholesterol metabolism as a consequence of HCV infection, key enzymes in the cholesterol biosynthetic pathway (Fig. 2) including rate-limiting HMG-CoA reductase (HMGCR) have been studied. In HCV core gene transgenic mice, hepatic transcription levels of HMGCR and HMG-CoA synthase (HMGCS) tended to be higher compared with controls, but the difference was not statistically significant.[97] Similar results were reported in JFH1-infected Huh-7 cells, in which transcription levels of HMGCR and squalene synthase appeared to be somewhat elevated; although statistical analysis was not performed because measurements were only made in duplicate.[103]

The above data were obtained from core gene transgenic mice livers and human hepatoma cell lines. However, cholesterol metabolism is subject to marked interspecies variation[104] and is very different between normal livers and hepatomas.[105] Therefore, the mRNA expression data in HCV-infected human liver reported by Nakamuta *et al.* are extremely valuable.[66,67] In their reports, transcription levels of HMGCR, HMGCS, farnesyl-diphosphate synthase and squalene synthase were all upregulated significantly in livers from chronic hepatitis C patients compared with controls. *De novo* cholesterol synthesis in the liver is mainly regulated by SREBP-2, which is synthesized in the endoplasmic reticulum and released as mature a transcription factor to the nucleus by sterol-sensitive proteolysis.[106,107] In Huh-7 cells transfected with NS4B, the protein expression levels of both precursor and mature forms of SREBP-2 were increased.[101] In contrast, hepatic transcription levels of SREBP-2 were not upregulated in core gene transgenic mice.[97] Therefore, NS4B rather than core protein may stimulate cholesterol biosynthesis in these models. In the livers from chronic hepatitis C patients, however, the upregulation of HMGCR is not associated with the transcription level of SREBP-2.[66] It is emphasized again that cholesterol metabolism is sometimes regulated differently among human and rodent livers, and the cultured human hepatoma cell line.

When hepatic transcription levels in chronic hepatitis C patients is compared with controls,[66,67] a striking abnormality in the regulation of cholesterol metabolism is pointed out, namely, upregulation of enzymes involved in the cholesterol biosynthetic pathway and marked downregulation of LDLR. Under physiological conditions, transcription of HMGCR, HMGCS, squalene synthase and LDLR are coordinately regulated by SREBP-2 because the 5′ flanking promoter regions of these four genes contain closely-related sequences designated as sterol regulatory elements.[108,109] In fact, significant positive correlations between HMGCR and LDLR mRNA levels have been observed in livers from chronic hepatitis C patients,[67] as well as in normal human livers.[110,111] The reason for the different transcriptional balance of hepatic HMGCR and LDLR between chronic hepatitis C patients and control subjects

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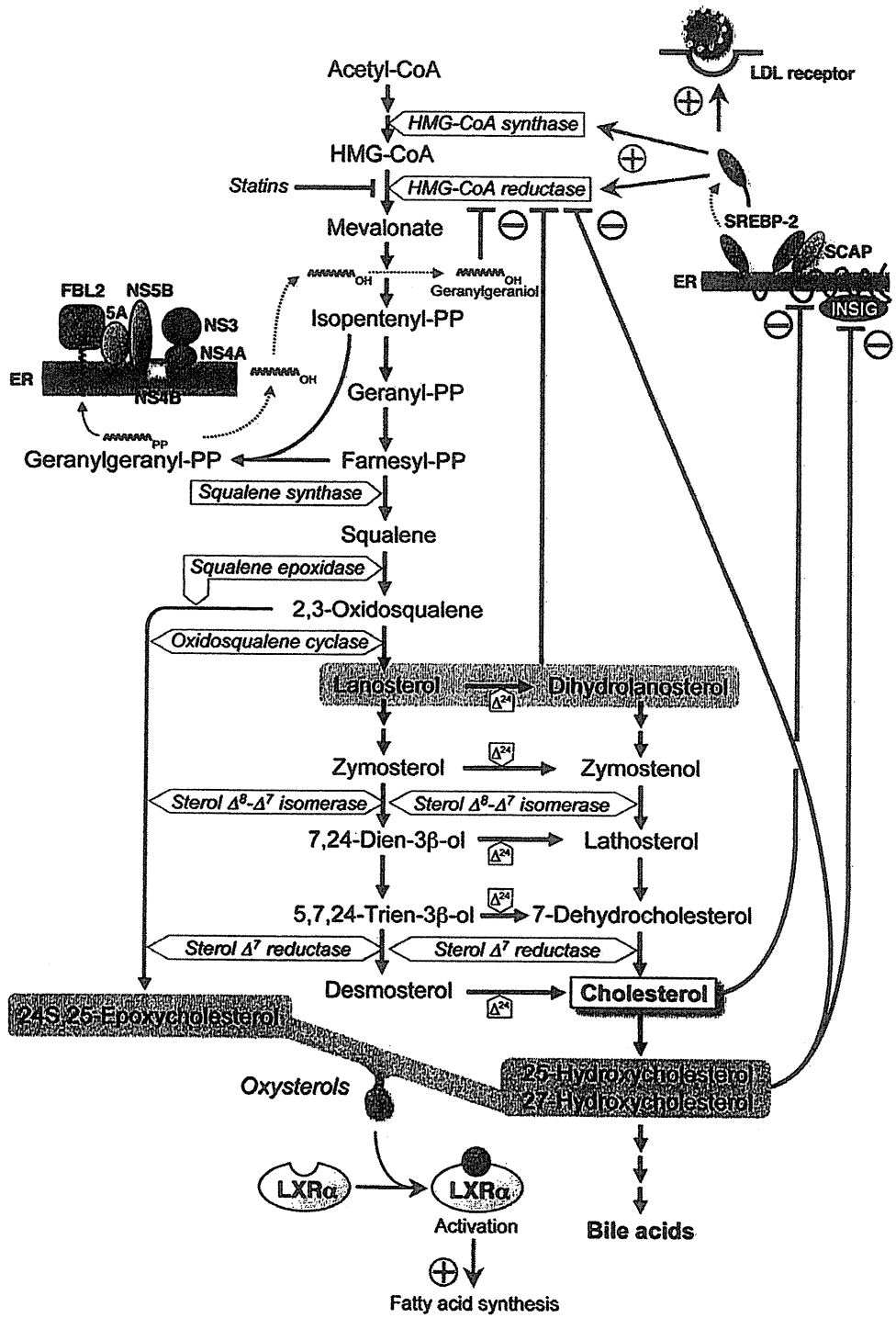

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