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Short communication

Mevalonate pathway modulation is associated with hepatitis C virus RNA presence in peripheral blood mononuclear cells

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ABSTRACT

Peripheral blood mononuclear cells (PBMC) constitute the main extrahepatic reservoir of hepatitis C virus (HCV). Lipid metabolism of host seems to play important role in HCV infection. The relationship between HCV presence in PBMC and the expression of mevalonate pathway has not been elucidated. The aim of this study was to investigate the association between mevalonate pathway and HCV RNA presence in PBMC after anti-HCV treatment. 67 serum and corresponding PBMC samples were collected from patients at the end of interferon alpha and ribavirin treatment. Serum total cholesterol, HDL-C and LDL-C fractions, triglycerides, as well as intracellular cholesterol and expression level of HMG-CoA reductase, geranylgeranyl pyrophosphate synthase in PBMC were measured and matched for the HCV RNA presence or absence in sera/PBMC. HCV RNA elimination from sera and PBMC was associated with higher serum cholesterol (118.5 mg/dL) and LDL-C (66.42 mg/dL) levels, compared to the group, where HCV RNA was detected only in PBMC (100.94 and 53.22 mg/dL) or the group, where HCV RNA was found in both sera and PBMC (86.79 and 43.79 mg/dL) after treatment. Increased expression of geranylgeranyl pyrophosphate synthase was found in the majority of PBMC samples that harbored HCV RNA after elimination of HCV RNA from sera. The expression of mevalonate pathway after antiviral treatment seems to be modulated depending on HCV RNA status in peripheral blood mononuclear cells.

High worldwide prevalence of Hepatitis C virus (HCV) infection (3%) remains significant health problem all over the world (Alter, 2007) providing major cause of chronic hepatitis that may progress to liver cirrhosis, and hepatocellular carcinoma (Levrero, 2006). HCV infection is mainly restricted to hepatocytes that play a vital role in human cholesterol homeostasis. Cholesterol is synthesized in hepatocytes via mevalonate pathway (Goldstein and Brown, 1990), under the control of rate limiting enzyme, 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase. The same pathway is responsible for synthesis of isoprenoids, farnesyl pyrophosphate and geranylgeranyl pyrophosphate that modify proteins in the prenylation process (Zhang and Casey, 1996). Recently, special attention has been focused on relation between host lipid metabolism, particularly mevalonate pathway, and HCV infection. Studies performed to date, suggest that chronic HCV infection is associated with decreased lipid level in sera of infected patients (Fabris et al., 1997; Siagris et al., 2006). On the other hand, the mevalonate pathway and its products seem to be directly involved in the life cycle of HCV. Both, HCV entry and replication require the presence of cholesterol-enriched plasma membrane microdomains in target cells (Aizaki et al., 2004; Barth et al., 2006). Moreover, Ye et al. (2003) reported that lovastatin, known inhibitor of mevalonate pathway, inhibited HCV RNA replication in HCV replicon–harboring hepatoma cells. Addition of geraniogeraniol can overcome the inhibitory effect of lovastatin on HCV RNA replication. Wang et al. (2005) identified that geranylgeranyl pyrophosphate (GGP) expression A in cells is necessary for geranylgeranylation of cellular protein FBL2 that in turn is indispensable for forming HCV replication complex.

Although the liver is the main place of HCV replication, gathered data indicate (Blackard et al., 2006; Zignego et al., 2007) that HCV can efficiently infect extrahepatic tissue, including peripheral blood mononuclear cells (PBMC). HCV RNA persistence in peripheral lymphoid cells after termination of antiviral therapy seems to be responsible for reinfection and reactivation of the disease. Despite the numerous studies of the relation between host lipid metabolism and HCV infection, the correlation between mevalonate pathway expression and HCV RNA presence in PBMC has not yet been elucidated. In the current study, we address this issue by analyzing the impact of post-treatment HCV RNA status in PBMC on the mevalonate pathway.

This study was approved by the local Review Board (RNN/33/03/KE). Blood samples were collected from 67 young, chronically infected patients (35 males, 32 females: ages 9–21) 1 month after

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Table 1
List of primers used in RT-PCR analysis includes sequences, size of PCR product and references.

Specificity of primers	PCR product (bp)	Sequence of forward and reverse primers	Source of primers
HCV RNA (external)	321	F:CCACCATGAATCACTCCCCTGT R:GCTCATGGTGCACGGTCTACGAGACCT	Dulak and Funk (1996)
HCV RNA (internal)	278	F:GTCTTCACGCAGAAAGCGTCTAGCC R:CACTCGCAAGCACCCTATCAGGCAG	
Beta-Actine	434	F:CAAAGACCTGTACGCCAACACA R:AACCGACTGCTGTCACCTTCAC	Delpuech et al. (2002)
HMG-CoA reductase	246	F:TACCATGTCAGGGGTACGTC R:CAAGCCTAGAGACATAATCATC	Powell and Kroon (1994)
GGPP synthase	124	F:GCCATGATTTTAACAGGTTTCC R:GAGAAATCCTCACCCAAGCA	UniSTS: 88997

antiviral therapy (IFN alfa2b + ribavirin). Serum and PBMC were isolated by blood centrifugation in density gradient (Histopaque 1077, Sigma). Serum total cholesterol (TC), HDL-C and LDL-C fractions, triacylglycerols (TG), as well as intracellular cholesterol (IC) and expression level of HMG-CoA reductase, geranylgeranyl pyrophosphate synthase in PBMC were measured and matched for the HCV RNA presence or absence in sera/PBMC. HCV RNA presence in sera was also determined 6 month after the completion of treatment. Total RNA was extracted from 1×10^6 PBMC by modified guanidinium/thiocyanate/phenol/chloroform technique. HCV RNA presence in sera was determined by RT-PCR (Cobas, Amplicor HCV 2.0 Monitor, Roche). HCV RNA in PBMC was detected by an in-house RT-PCR. For this, 6 μg of total RNA were reverse-transcribed and amplified by MasterAMP™ Tth DNA Polymerase (Epicentre® Biotechnologies) with external HCV-specific primers (Dulak and Funk, 1996) that are presented with others in Table 1. After first reaction (20 min of RT in 70 °C and 3 min in 94 °C followed by 35 cycles of 94 °C for 20 s, 50 °C for 20 s, 72 °C for 20 s and 72 °C for 7 min) the amplicon was used in the second-round PCR (2 min in 94 °C, 30 cycles of 94 °C for 40 s, 55 °C for 40 s, 72 °C for 40 s and 72 °C for 10 min).

Statistical analysis was conducted using Statistica Software (StatSoft). After data analysis by Kolmogorov–Smirnoff, differences in IC, TC, LDL-C, HDL-C, TG levels between groups were tested using repeated-measures ANOVA and the Student's *t*-test. The results of semi-quantitative RT-PCR analysis of gene expression were analyzed by Pearson's chi-square test. *P* values < 0.05 were considered statistically significant.

In the current study, we confirmed the post-treatment HCV RNA presence in 32 from 67 studied PBMC. In 35 cases called (only for the use of this study) 'responders' (R), sera and corresponding PBMC were free from HCV RNA. In 14 sero-negative cases, PBMC turned out to contain HCV RNA (group of 'responders with HCV RNA-positive PBMC', PR). In 18 cases HCV RNA was detected in both, sera and PBMC (group of 'non-responders', NR). Our results remain in line with others who showed that HCV RNA presence in PBMC was observed not only during chronic hepatitis but also after resolution of liver disease (Pham et al., 2004; Baré et al., 2005; Carreño et al., 2006). Only for 49 patients, we managed to test HCV RNA presence in sera 6 months after treatment. The sustained virological response (SVR) was achieved by 39 patients. As many as 32 'responders' were included in this group (plus 4 PR and 3 NR) whereas in the group that did not get SVR (10 cases) we found: only 1R, and 6 NR, 3 PR. For all patients we determined intracellular cholesterol (IC) in PBMC and lipid profile in sera at the end of anti-HCV treatment. Our analysis showed that (Table 2) patients, who eliminated HCV RNA from sera and PBMC, had significantly higher serum levels of total cholesterol (TC) and LDL-C compared to the group PR, whereas the lowest mean values of TC and LDL-C were found in group that harbors HCV RNA in both, sera and PBMC (NR). Mean values of TG and HDL-C in sera and IC in PBMC did not present the significant differences between groups.

The additional analysis based only on HCV RNA presence/absence in PBMC also demonstrated that mean levels of TC and LDL-C in sera corresponding to HCV RNA-positive PBMC were significantly lower compared to the group of responders (Fig. 1A). When the criterion was HCV RNA presence/absence in sera, we found (Fig. 1B) that HCV RNA presence in sera was additionally accompanied by significantly lower TG level (62.5 ± 27.5 mg/dL) compared to sera free from HCV RNA (96.55 ± 60.8 mg/dL). Study of Hamamoto et al. (2005) showed similar correlation, but between pretreatment TG level and HCV RNA in sera. Although our study was limited to the post-treatment observation, most of our results remain in line with earlier studies that demonstrated that lower cholesterol, LDL-C, apolipoprotein B levels before and after treatment (Minuk et al., 2000; Gopal et al., 2006; Backus et al., 2007) were associated with virus presence in sera. Moreover, for the first time, we managed to show the relationship between LDL-cholesterol level and HCV RNA status in PBMC. The influence of LDL-C fraction on HCV infection can be partially explain by the study of Monazahian et al. (1999) that revealed the importance of LDL-C particles in competitive inhibition of HCV binding to the LDL receptor on the surface of different human cells in vitro. We can speculate that also in case of our study, observed high level LDL-C in sera might be responsible for inhibition of HCV binding to LDL receptors on PBMC membrane and in turn leads to restriction on HCV propagation in PBMC in vivo.

It was checked that type of response as well as serum lipid profile was not related to patients' age and gender. Genotype determination, although performed in part of patients (29 from 67), showed that only genotype 1 was presented in all tested samples. It remains in accordance with earlier polish epidemiological studies

Table 2
Comparison between lipid profile and the type of antiviral response.

Lipid profile (mg/dL)	Three types of antiviral response			*p*-Value
	R (*n* = 35)	PR (*n* = 14)	NR (*n* = 18)	
IC	152.9 (±73.1)	130.6 (±43.0)	125.3 (±32.3)	0.3
TC	118.5 (±34.4)	100.9 (±24.6)	86.8 (±30.3)	0.007
IC/TC	1.39 (±0.4)	1.34 (±0.4)	1.47 (±0.3)	0.7
LDL-C	66.4 (±24.3)	53.2 (±16.2)	43.8 (±18.5)	0.004
HDL-C	31.5 (±10.1)	30.0 (±7.8)	29.6 (±11.8)	0.8
TG	99.2 (±69.9)	91.9 (±42.2)	62.5 (±27.5)	0.1

Note: Mean levels of: intracellular cholesterol (IC) in PBMC, total cholesterol (TC), IC/TC ratio, LDL-C, HDL-C fractions and triacyglycerols (TG) in groups, PR, NR. IC was tested by cholestrol Chod-PAP, cholesterol assay kit (BIOLABO S.A., France) as per the manufacturer's recommendations. Results were normalized according to the protein level in cell lysates. Protein concentration in all cell lysates was determined using Bio-Rad Protein Assay (Bio-Rad). TC, HDL-C, LDL-C, TG levels were measured enzymatically with commercial kits (OLYMPUS) in an automated analyzer Olympus AU 400.