Research Article

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PTEN protein phosphatase activity regulates hepatitis C virus secretion through modulation of cholesterol metabolism

Marion Peyrou[2,†], Sophie Clément[1,†], Christiane Maier[1], Lucie Bourgoin[2], Emilie Branche[1], Stéphanie Conzelmann[1], Vincent Kaddai[1], Michelangelo Foti[2,†], Francesco Negro[1,3,*,†]

[1]*Division of Clinical Pathology, University Hospital, University of Geneva, Geneva, Switzerland;* [2]*Department of Cellular Physiology and Metabolism, Faculty of Medicine, University of Geneva, Geneva, Switzerland;* [3]*Division of Gastroenterology and Hepatology, University Hospital, University of Geneva, Geneva, Switzerland*

Background & Aims: Hepatitis C virus (HCV) infection is dependent on lipid metabolism. Hepatocyte steatosis occurs frequently in HCV infection, but the relationship between steatosis and HCV life cycle is unclear. We showed that HCV induces steatosis via the downregulation of the phosphatase and tensin homolog deleted on chromosome 10 (PTEN). We here investigated how PTEN may affect HCV production.

Methods: The effect of overexpression or silencing of PTEN on HCV secretion was assessed in genomic-length Jc1 infected HuH7 cells. The role of PTEN protein and lipid phosphatase activities on lipid metabolism and infectious viral particle secretion was investigated using dominant-negative PTEN mutants. The importance of cholesterol metabolism for PTEN-dependent lipid droplet biogenesis and viral particle secretion was examined using statins.

Results: *PTEN* silencing in Jc1 infected HuH7 cells stimulated HCV particle secretion, while PTEN overexpression decreased virus egress. Viral secretion was also increased by overexpression of protein phosphatase-deleted ($PTEN_{Y138L}$), but not lipid phosphatase-deleted ($PTEN_{G129E}$), PTEN mutant, thus indicating that the protein phosphatase activity of PTEN controls viral secretion. Similarly, $PTEN_{Y138L}$, but not $PTEN_{G129E}$ mutant induced the formation of large lipid droplets. $PTEN_{Y138L}$ mutant did not affect biosynthesis of triglycerides, but promoted the biosynthesis of cholesterol esters. Consistently, statins prevented the increased cholesterol ester production, large lipid droplet formation, and viral secretion in cells expressing the $PTEN_{Y138L}$ mutant.

Conclusions: Downregulation of PTEN protein phosphatase activity by HCV affects cholesterol metabolism, thereby inducing the appearance of large lipid droplets and increasing virion egress.

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Introduction

The hepatitis C virus (HCV) infection is a major cause of chronic liver disease worldwide. The progression of chronic hepatitis C towards cirrhosis and hepatocellular carcinoma (HCC) is accelerated by several host and viral factors [1]. Among these, steatosis is the focus of major research, not only because it is a hallmark of the metabolic syndrome, but also because HCV directly affects lipid metabolism [2], thereby favouring its own replication, morphogenesis, and secretion [3]. Although steatosis, i.e., an excess neutral fat stored in large lipid droplets (LD) in the hepatocyte cytoplasm, occurs frequently in HCV infection, its role (if any) in the viral life cycle remains unclear. During the HCV polyprotein synthesis, the core protein relocates from the endoplasmic reticulum to the surface of LD [4], an event suggested to promote virion assembly [5]. Then, HCV exploits the very low density lipoprotein secretory pathway to exit hepatocytes in the form of lipid-rich infectious particles [6]. Moreover, inhibitors of lipid synthesis, such as statins, decrease viral replication *in vitro* [7,8] and in patients [9]. Thus, HCV replication, virion assembly, and egress are intricately linked to lipid metabolism, but whether the appearance of large LD favors HCV life cycle is unclear.

Although the mechanisms of HCV-induced steatosis are multiple [10], we reported that the core protein of HCV genotype 3 causes the accumulation of large LD by downregulating PTEN (phosphatase and tensin homolog deleted on chromosome 10) [11]. PTEN is a ubiquitous lipid (phosphoinositide [PI]) and protein phosphatase considered as a major tumour suppressor, being frequently mutated or deleted in several human cancers, including HCC [12]. Through its lipid phosphatase activity, it acts as a potent negative regulator of the PI-3 kinase (PI3K) pathway [13]. In contrast, the functional role of PTEN protein phosphatase

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* Corresponding author. Address: Divisions of Clinical Pathology and of Gastroenterology and Hepatology, University Hospital, 4 rue Gabrielle-Perret-Gentil, 1211 Geneva 4, Switzerland. Tel.: +41 22 3795800; fax: +41 22 3729366.
E-mail address: Francesco.Negro@hcuge.ch (F. Negro).
† These authors contributed equally to this work.

Abbreviations: PTEN, phophatase and tensin homolog deleted on chromosome 10; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; LD, lipid droplet; PI, phosphoinositide; NAFLD, non-alcoholic fatty liver disease; CE, cholesterol ester; ORO, Oil-Red-O; TG, triglyceride.

activity is debated, although it may be important to regulate its own activity and localization and for cellular adhesion processes [14–16]. Liver-specific *PTEN* knockout mice sequentially develop steatosis, steatohepatitis/fibrosis and HCC [17], and, interestingly, PTEN is downregulated in the liver of patients with non-alcoholic fatty liver disease (NAFLD) [18] and HCV-induced steatosis [11]. Thus, PTEN downregulation appears to be clinically relevant, as it may contribute to liver disease progression in patients with NAFLD and hepatitis C. Whether PTEN effects on lipid metabolism and LD formation may also affect HCV life cycle is unknown.

Here we show that the alteration of PTEN expression, and more specifically of its protein phosphatase activity, in the context of HCV infection, leads to an increased synthesis of cholesterol esters (CE) and of LD size, accompanied by an enhanced secretion of infectious viral particles.

Materials and methods

Primers, antibodies, plasmids, and reagents

Reagents, primers, antibodies, and plasmids are described in Supplementary Table 1.

Real-time PCR, immunoblot analysis, and lentivirus production

RNA isolation, reverse transcription, real-time PCR, immunoblot analysis, and lentivirus production are described in Supplementary Materials and methods.

In vitro transcription

Chimeric JFH1-J6 (Jc1) full length RNA was generated from pFK-J6/C3 (Jc1) (from R. Bartenschlager, Heidelberg, Germany), by using the T7 Ribo Max Express Large scale RNA Production System (Promega). *In vitro* transcripts were purified using the Nucleospin RNAII kit (Macherey Nagel), and their integrity verified using the Agilent 2100 bioanalyzer.

Virus production, titration, and infection

To produce infectious particles, HuH7.5 cells (7.5×10^6) were electroporated with 10 μg of HCV RNA transcribed from pFK-J6/C3 (Jc1) in Cytomix [19] using a Gene Pulser II electroporator (260 V, 950 μF; Bio-Rad Laboratories). Culture supernatant was harvested after 48 h, filtered through 0.45 μm pore-sized PVDF membrane, and titered by infecting HuH7.5 cells by serial dilutions. Cells were fixed after 48–72 h with −20 °C methanol and immunostained using an anti-core (C7-50) antibody [20]. $TCID_{50}$/ml was calculated as reported [21]. HuH7 cells were routinely infected with 1–2 MOI for 48 h.

Immunochemistry and Oil-Red-O staining

Paraformaldehyde-fixed cells were immunostained using the anti-core antibody [20]. Neutral lipids were stained with Oil-Red-O (ORO). Images were acquired with an Axiophot photomicroscope equipped with an Axiocam camera (Zeiss). Surface area of individual LD was calculated using the Metamorph software (Molecular Devices Corporation).

Triglyceride and cholesterol ester measurement

Lipids were extracted using a technique adapted from the Folch procedure: methanol/chloroform (1/2 v/v) were added to dried cell pellets. After 1 h incubation at RT, 250 μl H_2O was added prior to centrifugation at 2000 rpm. The lower phase was collected and dried overnight at RT. TG and CE were measured using the GPO/PAP kit (Roche) and the cholesterol/cholesteryl ester quantitation kit (Calbiochem), respectively.

Statistical analysis

Results were expressed as mean ± SEM of at least three independent experiments, and analyzed by Student *t*-test or *z*-test when n >30. Values of $^{***}p$ <0.001, $^{**}p$ <0.01, and $^{*}p$ <0.05 were considered statistically significant.

Results

HCV infection decreases PTEN protein expression through post-transcriptional mechanisms

We have previously shown that HCV 3a core protein induces a downregulation of PTEN protein, but not mRNA, in HuH7 cells, suggesting a post-transcriptional control of PTEN expression [11]. Jc1 infection of HuH7 cells also led to a significant reduction of PTEN protein expression, but not of its mRNA (Fig. 1A and B). However, the expression of the Jc1 core protein alone did not downregulate PTEN, suggesting that other viral proteins encoded by Jc1 are involved in this process (Fig. 1A).

PTEN controls HCV particle secretion

To evaluate whether PTEN downregulation may impact HCV life cycle, we established HuH7 cell lines through lentiviral transductions expressing *PTEN* shRNAs or overexpressing PTEN. Cells infected with Jc1 HCV particles were analyzed two days post-infection. Both overexpression and depletion of PTEN slightly decreased the intracellular content of the HCV core protein (Fig. 1C), but failed to affect the efficiency of Jc1 infection, as assessed by immunostaining for HCV core protein (Fig. 1D), or by measuring the level of intracellular HCV RNA by real-time PCR (Fig. 1E).

We then assessed whether modulating PTEN expression levels may affect HCV release and infectivity, measured, respectively, as extracellular HCV RNA level and $TCID_{50}$/ml of culture supernatants. PTEN overexpression reduced virion secretion and infectivity by about 50%, while *PTEN* silencing stimulated both virion secretion and infectivity by more than two-fold (Fig. 1E and F). These data indicate that the secretion efficiency of HCV particles is inversely correlated with PTEN expression and that PTEN downregulation stimulates HCV egress.

PTEN protein, but not lipid, phosphatase activity restrains HCV egress

Two enzymatic activities have been reported for PTEN: a protein and lipid phosphatase activity. To investigate which of these two activities may affect HCV secretion, we engineered lentivectors encoding three PTEN mutants previously described to be deficient for the protein phosphatase activity only ($PTEN_{Y138L}$) [15], for the lipid phosphatase activity only ($PTEN_{G129E}$) [22], or for both activities ($PTEN_{C124S}$) [23]. HuH7 cells were then transduced with these mutated proteins that behave as dominant negative mutants over the endogenous PTEN [24]. As expected, and consistent with previous reports [15], expression of PTEN mutants impaired in its lipid phosphatase activity ($PTEN_{G129E}$ and $PTEN_{C124S}$) displayed an increased constitutive and insulin-induced phosphorylation of Akt, while the mutant deficient for the protein phosphatase activity only ($PTEN_{Y138L}$) did not (Supplementary Fig. 1).

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