Role of HCV Capsid Protein on Cellular Lipid Droplets Content and Localization during HCV Infection

Muhammad Sohail Afzal, Najam us Sahar Sadaf Zaidi, Jean Dubuisson, and Yves Rouill é

Abstract—**Hepatitis C virus (HCV) infects approximately 3% of the world's population, with more than 10 million chronically infected persons in Pakistan; establishing chronic infection in the majority of cases. HCV life cycle and lipid metabolism are tightly linked, resulting in steatosis for many patients. As lipid droplets (LDs) have emerged as crucial cellular organelles, which are necessary for persistent viral propagation and virion production, the study was designed to evaluate the role of HCV core protein in lipid droplets morphology and lipid metabolism disruption. Huh-7 cells were transfected with core expression vectors and the effect of core on cellular LDs was monitored by confocal microscopy. Core protein was localized on LDs surface and interfered with lipid droplets morphology. Core protein after localization on LDs surface increased intracellular lipid content. Lipid contents in cells expressing wild type core was higher as compared with cells transfected with a mutated core (double mutant P138A, P143A), which is deficient for LDs localization. Attachment of core to LDs induced a redistribution of LDs. The redistribution induced by core protein aggregated LDs around the nucleus in HCV-transfected cells, in a manner very similar as during HCV infection. The study confirms the role of HCV core protein in the disruption of lipid metabolism and in the redistribution of LDs during viral infection, which might be a pathway for HCV persistence and pathogenesis.**

Index Terms—**Core, HCV, lipid droplets, steatosis.**

I. Introduction

Hepatitis C is a major public health concern worldwide. It is caused by hepatitis C virus (HCV), a small hepatotropic virus infecting 170-180 million people worldwide [1]. Globally 0.25-1.25 million new cases of HCV infection have been reported per year [2]. In Pakistan more than 10 million people are suffering from chronic hepatitis C with a very high mortality and morbidity rate [3]. HCV is a major causative agent for chronic hepatitis, cirrhosis, and hepatocellular carcinoma.

HCV is a small enveloped, plus stranded RNA virus and is a member of the family Flaviviridae. Its RNA genome encodes single open reading frame, which is translated into a polyprotein of more than three thousand amino acid residues. Viral and cellular proteases cleave the polyprotein co- and posttranslationally into 10 individual proteins. Viral genome has three structural proteins (core, the capsid protein, and two envelope glycoproteins E1 and E2) and seven non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) proteins, which are involved in viral RNA replication and virion assembly [4]. HCV plays a very important role in hepatic steatosis by increasing body mass index and visceral obesity. It decreases serum cholesterol level, low-density lipoprotein cholesterol (LDL) and apolipoprotein B (apoB) [5].

Lipid droplets (LDs) are cellular lipid storage organelles. LDs have attracted considerable attention because of their accumulation during human diseases such as obesity, atherosclerosis, and HCV-associated liver malfunctions [6]. It was reported that LDs are directly involved in the production of infectious HCV particles [7]. In HCV-infected cells, core is localized on the surface of LDs. The mature form of core is a dimeric, □ -helical protein, which is composed of two domains, D1 and D2 [8], [9]. D2 is responsible to target core to LDs [9]. Mutational analysis showed that substituting two proline residues (P138 and P143) in D2 by alanines blocked the core localization on LD surface [10]. The aim of the study was to evaluate the role of core on lipid biogenesis and LDs localization during HCV infection.

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II. Materials and Methods

A. Chemicals

Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), geneticin, goat and fetal calf sera (FCS), BODIPY 493/503, and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Life Technologies.

B. Antibodies

Mouse monoclonal anti-core antibody ACAP27 was kindly provided by J.-F. Delagneau (BioRad, France). Cy3-conjugated goat anti-mouse was purchased from Jackson Immunoresearch (West Grove, PA).

C. Cell Culture

Human hepatoma-derived cells (Huh-7) were grown in Dulbecco's modified Eagle's medium. The media was supplemented with 10% fetal calf serum and 10 μg/ml gentamicin.

D. Plasmids

To construct the core expression vector, a cDNA encoding residues 1 to 191 of the polyprotein was amplified by PCR from the plasmid pJFH1-CSN6A4, and subcloned into the expression vector pCIneo (Promega) between a Kozak

consensus sequence and a stop codon. To produce an expression vector for the core PP mutant, the codons of residues P138 and P143 were replaced with alanine codons by overlapping PCR. Both constructs were confirmed by DNA sequencing

E. Transfection

Approximately 24 h prior to transfection, Huh-7 cells were seeded on glass cover slips. Next day, transfection was done with 50-60 % confluent cells, by using the following protocol: 25 µl of OptiMEM media was mixed with 2 µl of transfection reagent TransIt-LT1 (Mirus) and incubated at room temperature for five minutes. 0.5 µg of core-expression plasmid was then added and the mix was further incubated for 30 min. The transfection mix was then added drop wise to freshly added cell culture medium (0.5 ml), then mixed with a pipette and incubated at 37 ℃.

F. Immunofluorescence Microscopy

Huh-7 grown on cover slips were washed with PBS and fixed with 3% PFA for 25 min. Cells were then permeabilized for 3 min with 0.1% triton X-100, blocked with 10% goat serum, and labeled for 30 min with core-specific mouse monoclonal antibody. After three PBS washes, cells were incubated with Cy3-conjugated goat anti-mouse IgG antibody and DAPI to label nuclei. A 10-min incubation with 0.2 µg/ml BODIPY 493/503 was used to stain LDs.

Images were acquired with an LSM710 laser-scanning confocal microscope (Zeiss) using a 63X/1.4 numerical aperture oil immersion objective. Signals were sequentially collected by using single fluorescence excitation and acquisition settings to avoid crossover.

III. Results

Huh-7 cells were transfected with plasmids expressing wild type core, or a core PP mutant (P138A, P143A double substitution) with a reduced interaction with LDs. Cells were then double-labeled to detect core expression and LDs. A shown in Fig. 1, core was co-localized with LDs. The size of LDs in core-expressing cells was increased, compared to control cells with no core expression (Fig. 1), indicating that core protein increases cellular LD content. In contrast, core PP mutant had no apparent impact on LD size or number (fig 2). As expected this mutant did not co-localize with LDs (fig 2). This suggests that core protein interferes with lipid metabolism, and that this action of core requires core to be associated with LDs.

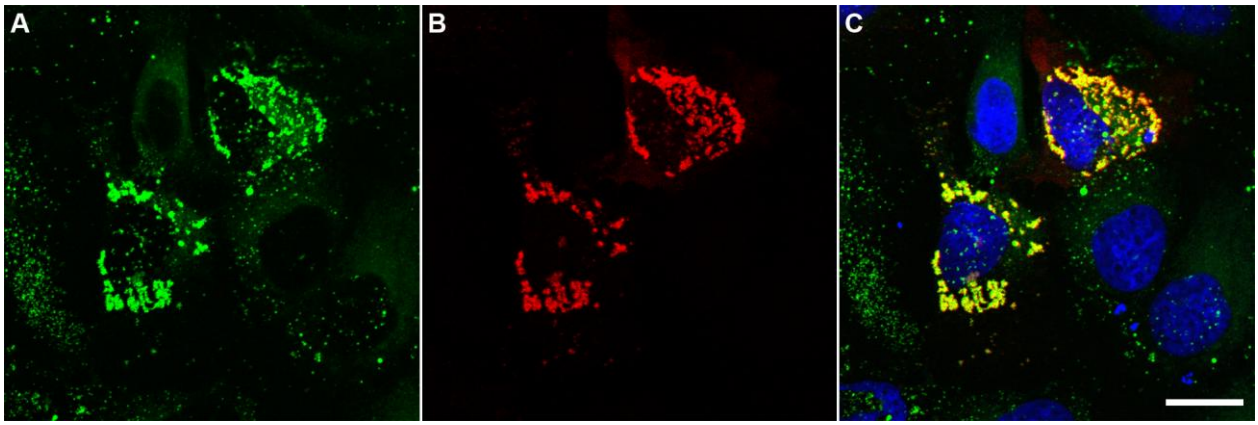

Fig. 1. Immunofluorescence analysis of Huh-7 cells expressing core. Huh-7 cells grown on glass cover slips were transfected with a core-expressing plasmid, and processed for fluorescent detection of cellular LDs (A) and core (B). A merged image of channels A and B with nuclei in blue is shown in C. Bar, 10 µm.

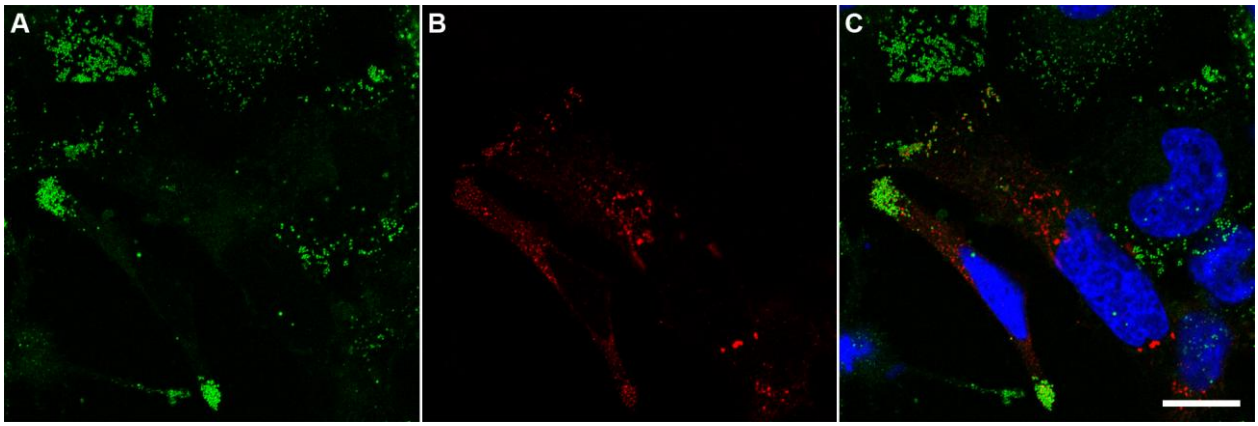

Fig. 2. Immunofluorescence analysis of Huh-7 cells expressing core PP mutant. Huh-7 cells grown on glass cover slips were transfected with a plasmid expressing a core mutant with reduced association with lipid droplets, and processed for fluorescent detection of cellular LDs (A) and core (B). A merged image of channels A and B with nuclei in blue is shown in C. Bar, 10 µm.

In addition to the increase of cellular content of neutral lipids, evidenced by the dramatic increase in LD mean size, core expression had also an impact on the intracellular localization of LDs. In cells expressing wild-type core, LDs were located around nucleus, whereas control cells with no core displayed smaller LDs scattered throughout the whole cytoplasm of the cell (Fig 1). Again, this action of core was not detected in cells expressing core PP mutant (Fig. 2).

IV. Discussion

Lipids play an important role during HCV infection. HCV virions circulating in patient serum are associated with lipoproteins. Moreover, steatosis was frequently observed in HCV patients [11]. Steatosis results from a severe accumulation of neutral lipids, such as triglycerides, in hepatocytes. Inside cells, neutral lipids accumulate in LDs, which have been shown to be essential cellular organelles for the viral life cycle. It was established that the association of core with LDs coincides with virus particle release and that disrupting this association reduces the production of infectious viral particles [12]. The results of the current study confirmed the association of core with LDs, and that LDs and core interaction may interfere with the cellular lipid metabolism, resulting in an increased cellular content of neutral lipid. The results also suggested that the interaction of the capsid protein of HCV with LDs induces a redistribution of these cellular organelles In core-positive cells LDs were localized in a perinuclear area, while in normal cells, LDs were much more evenly distributed in the whole cytoplasm.

It was shown that domain 2 of core is required for directing core on LD surface. During the course of an HCV infection, core was reported to progressively coat the entire LD surface over time [12]. In the same time, core also aggregates LDs around the nucleus. LDs biogenesis and redistribution induced by core may disturb their normal function. Results of in vivo animal studies have shown that core expression in transgenic mice liver can induce steatosis (LD accumulation) and prevent release of very low-density lipoprotein (VLDL). VLDL assembly relies on lipid provided by LDs [13], [14]. The other possible mechanism of intracellular lipid accumulation in core-transfected cells may be the inhibition of microsomal triglyceride transfers protein activity by HCV core protein. This enzyme plays a role in lipid transfer to VLDL. Hence HCV core mediated microsomal triglyceride transfers protein inhibition leads to decreased hepatocyte lipid export and may also contribute to hepatic steatosis [15]. It was also observed that in core-transfected cells transcription factors that induce the expression of genes involved in lipogenesis, cholesterologenesis and sterol-regulatory element binding protein-1 (SREBP) mRNA expression were higher and that this results inincreased fatty acid synthesis in core-expressing hepatocytes [16].

Our findings are important for understanding the role of HCV in the development of pathologies, such as steatosis, which is a very common phenomenon in chronically infected patients. HCV capsid protein may interfere with lipid biogenesis, and export from hepatocytes and this would result in accumulation of lipids in infected cells, which may lead to steatosis. Disruption of normal lipid metabolism in infected cells may lead to complications in HCV-infected patients.

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