Published in final edited form as:

Virus Res. 2009 January ; 139(1): 127–130. doi:10.1016/j.virusres.2008.10.008.

Recombinant Adenoviruses Expressing Steatosis-Associated Hepatitis C Virus Genotype 3 Core Protein Produce Intracellular Lipid Accumulation in Cultured and Primary Hepatocytes

Guan Qiang¹, Liu Yang², Rafal P. Witek², and Ravi Jhaveri¹

¹*Division of Infectious Diseases, Department of Pediatrics, Duke University Medical Center, Durham, NC 27710*

²*Division of Gastroenterology, Department of Medicine, Duke University Medical Center, Durham, NC 27710*

Abstract

Steatosis, or “fatty liver”, is a common finding in Hepatitis C virus (HCV) infection and reflects dysregulation of lipid homeostasis. HCV Core protein causes lipid accumulation when expressed in cultured cell lines, which data now indicates is a critical step for HCV replication, assembly and release. Previous studies on HCV Core and lipid accumulation have been limited by poor efficiency of conventional transfection techniques or the cell line limitations of the available cell culture system. In this study, we have designed recombinant adenoviruses expressing HCV Core and demonstrate that infection of both cultured and primary cells leads to intracellular lipid accumulation. This system will allow for detailed studies of the mechanisms that drive intracellular lipid accumulation during HCV infection and addresses some of the disadvantages that have hampered previous research.

Keywords

Hepatitis C virus; Core protein; Steatosis; Recombinant Adenoviruses; Lipid; Primary Hepatocytes

Steatosis, or “fatty liver”, is a common finding in Hepatitis C infection.[1] Approximately half of all patients have evidence of steatosis on liver biopsy, but that figure climbs to 80% when examining patients with genotype 3 infection.[1,2] Steatosis observed in genotype 3 appears to be linked to viral activity and specific viral sequences within the Core protein.[1,3] This is in contrast to other genotypes that correlate more with host susceptibility factors like diabetes, obesity and alcohol use.[1,2]

Hepatitis C virus (HCV) almost exclusively infects the liver, and recent data using the only available tissue culture system has shown that HCV utilizes a unique mechanism for viral replication, assembly and release that involves lipid droplets.[4] The Core protein binds to lipid droplets, subsequently recruits non-structural protein NS5A and HCV RNA and then utilizes the very-low density lipoprotein secretion pathway to release fully assembled viral particles.

Corresponding Author: Ravi Jhaveri M.D., Division of Pediatric Infectious Diseases, Duke University Medical Center, DUMC 3499, Durham, NC 27710, Tel: 919-684-6335, Fax: 919-668-4859, Email: ravi.jhaveri@duke.edu.

Conflicts of Interest: The authors have no conflicts to report.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

[4-7] Core protein also causes lipid to accumulate when expressed in vitro in a variety of cell types.[2,3,8-10] Putting these facts together, it is likely that Core protein causes lipid accumulation as part of its life cycle in order to assemble infectious virus, and that steatosis is the end result of this lipid accumulation over time.

In previous studies, we identified specific polymorphisms within the genotype 3 HCV Core protein that were associated with steatosis.[3] When expressed in vitro, these steatosis-associated isolates caused an increased amount of lipid to accumulate compared to non-steatosis clones. One of the challenges we encountered in those studies was a low expression level with conventional transfection techniques. In order to perform more detailed studies investigating mechanisms of lipid accumulation, we sought to design a system where we could achieve very high expression levels in both cultured and primary cells. We chose to use recombinant adenovirus technology because it satisfies all of the previously mentioned criteria. In this report, we describe the design and application of this method of expressing HCV Core protein that will facilitate future studies of lipid accumulation.

We began by cloning 2 isolates from our previous study, HCV1 and HCV12 (GenBank accession #EU099414 and EU099417) as the template for cloning.[3] We used the exact same primers, cloning methods and PCR conditions as described in our previous paper except that a *Bgl*III site was substituted in the forward primer and an *Xho*I site was substituted in the reverse primer of our custom designed oligonucleotides for HCV Core genotype 3.

We cloned into the pShuttle-IRES-hrGFP-2 vector (AdEasy system, Stratagene, La Jolla, CA), and subsequently transformed into the BJ5183 Cells to produce Recombinant AdEasy Plasmids. Individual positive recombinant adenovirus plasmids, identified by restriction digest using *Pac*I, were used to transform competent cells. Following the manufacturer's protocol, we amplified the adenovirus preparation several times using Ad293T cells to increase the titer of virus. We subsequently used the purified adenovirus stocks to perform infections and analyze protein expression and lipid accumulation.

Origin and maintenance of 5H cells were previously described.[3,11]. Cells were infected with an estimated 100 viral particles/cell in serum-free DMEM media at 37°C, with replacement with FBS containing media after 2 hours. At 24 hours, cell lysates were prepared using Passive Lysis Buffer (Promega, Madison, WI) and analyzed by immunoblot with anti-Core antibody (Austral, San Ramon, CA).

Animal care and procedures were approved by the Duke University Medical Center Institutional Animal Care and Use Committee as set forth in the 'Guide for the Care and Use of Laboratory Animals' published by the National Institutes of Health. Primary hepatocytes were isolated from adult C57Bl-6 mice using a protocol involving perfusion and collagenase digestion outlined previously.[12] Cells were collected and centrifuged, viability was determined using trypan blue staining and subsequently plated in 4 well chamber slides. Cells were initially incubated in DMEM media containing 10% fetal bovine serum, 1% penicillin/streptomycin, insulin and dexamethasone. Regular media (without insulin and dexamethasone) was used once cells demonstrated attachment to slides. Infections were performed 1-2 days after isolation to assure viability throughout the experiments.

Infections were performed with an estimated 100 viral particles/cell. Virus stocks were mixed in DMEM media containing 25 mM HEPES alone. Virus containing media was applied to cells for 2 hours, after which cell specific serum containing media was added. Cells were incubated for 24 hours prior to staining.

Procedures for Immunofluorescence and Oil Red O staining were performed as described previously.[3] Slides were examined using Axiovert 200 microscope (Carl Zeiss) with

epifluorescent illumination, and images recorded using an AxioCam HRC camera (Carl Zeiss) and Axiovision 4.5 software (Carl Zeiss).

We quantified differences in lipid accumulation between our recombinant adenoviruses using methods we have previously described and validated.[3] Twenty 100x fields were examined for each set of infections in primary cells. Image analysis was performed using MetaMorph version 7 software (Molecular Devices, Downingtown, PA) To summarize, a region was drawn around each cell or cells expressing GFP or HCV Core. The region was transferred to the same coordinates on the corresponding brightfield image. A red threshold was applied and % area thresholded was recorded. Mean % area thresholded red and standard deviations were calculated for GFP and HCV Core and statistical analysis was performed using open source programs (www.openepi.com).

We used 5H cells in our initial experiments because of the low background levels of lipid and HCV Core induced lipid accumulation and co-localized to lipid droplets in our initial experiments[3]. After amplifying and purifying the adenovirus stocks, we infected 5H cells and collected cell lysates after 24 hours. These were analyzed using western blot and found to have a band of the appropriate size for HCV Core (data not shown).

5H cells were passaged into chamber slides and after 24 hours were infected with either no virus, GFP containing virus or our steatosis-associated HCV Core protein containing virus. After 24 hours, the cells were washed, fixed and stained with HCV Core antibody followed by a FITC-conjugated secondary antibody and then Oil Red O (ORO) stain. The results are shown in Figure 1. Cells not infected with virus had minimal levels of intracellular lipid (not shown), as did the cells infected with GFP containing virus. Cells infected with virus containing HCV Core had numerous lipid droplets, and the fluorescence pattern showing Core co-localizing with lipid droplets. Both of these findings are consistent with our previous observations using conventional transfection techniques. We also found that 30-40% of cells expressed Core protein, which far outnumbers those we observed in transfection experiments.

Given that these HCV Core expressing adenoviruses produced the same localization and lipid accumulation in a cultured liver cell line as when we used transfection techniques, we tested whether infection of freshly isolated primary hepatocytes would produce the same or similar results.

After perfusion of mouse liver, the hepatocyte cell fraction was isolated and plated in 4 well chamber slides. After 24 hours, cells were mock infected, infected with either GFP adenovirus or HCV Core expressing adenovirus. Similar to the cultured cell line, we achieved 30-40% efficiency and the results of the ORO staining are shown in Figure 2a. Cells infected with GFP virus had few lipid droplets that were small in size with bright green fluorescence. Cells expressing HCV Core had dramatically increased lipid content, well above the amount observed in the GFP infected cells, with much larger lipid droplets. Figure 2b shows the results of the Metamorph analysis of ORO content. The cells expressing HCV Core protein had significantly higher ORO compared to GFP expressing cells (40% vs. 12%, $p<0.0001$). Additionally, we observed the same co-localization of Core protein with the lipid droplets as seen in Figure 3.

These findings reproduce results using conventional transfection techniques in cultured cell lines except with much higher efficiency and the ability to use primary hepatocytes which are very difficult to transfect[3]. The observation that Core protein co-localizes to lipid droplets in primary hepatocytes provides validation of this system since recent work using the HCV JFH-1 cell culture system has shown that this interaction serves to facilitate the recruitment of NS5A and then newly transcribed viral RNA, which ultimately leads to the assembly and release of new viral particles[4-7].

This system provides several advantages compared to previous small animal studies and the currently available tissue culture system. Most of the small animal work on steatosis has been with transgenic animals that constitutively express HCV Core protein. Given the effort and manipulation it takes to breed these animals, this raises issues of cost, time and generalizability of the results[13]. The JFH1 tissue culture system continues to provide breakthrough data on details of the HCV viral replicative process and intracellular life cycle, but it is important to acknowledge the many limitations of the system[14]. First is that it must be generated in a highly selected cell line, Huh7.5 cells, which display high levels of lipid at baseline. Studies of lipid accumulation in these cells are extremely difficult. Second, it represents only one genotype that causes a small minority of infections. While it is true that the structural region of the virus is flexible and will allow some substitution of genes from other genotypes, only recently has a genotype 3a chimera produced significant titers.[15] Third, studies in primary cells and small animals have yet to be performed since human or chimpanzee CD81 is required for entry. Our system will allow us to examine primary cells from wild type small animals or even scavenged human tissues to perform some comparative analysis on mechanisms of lipid accumulation.

One caveat of the system is worth mentioning. We do not have a way to directly compare protein expression levels between our system and conventional techniques. We have performed immunoblotting analysis (data not shown) that show comparable band intensities, but this is not a quantitative result. This is an issue we plan to investigate further as we move forward.

We have 2 major directions planned for future investigation. The first is performing “metabolomic” analyses in primary hepatocytes. Given that cultured hepatocyte cell lines are frequently derived from hepatocellular carcinomas and may not be completely representative of the biochemistry within normal hepatocytes, being able to express HCV Core protein at high levels will allow us to study which species of lipid is accumulating and the specific pathways involved. The second direction is moving toward *in vivo* studies. Using small animal models, we can begin to perform dynamic studies not only of the genesis of steatosis, but also examine specific factors of host behavior and susceptibility, including obesity, diabetes and alcohol consumption. We can additionally design HCV Core adenoviruses from other genotypes in order to compare directly how the combination of host and viral factors contribute to lipid accumulation and steatosis formation.

In summary, we have developed recombinant adenoviruses expressing steatosis-associated HCV Core protein. These adenoviruses recapitulate lipid accumulation in cultured and primary hepatocytes. We will use this model to further our studies related to the mechanisms of lipid accumulation.

Acknowledgements

The authors would like to thank Anna Mae Diehl M.D. and members of the Diehl laboratory for their assistance and support of this work.

Funding Support: RJ: NIH K08DK76598, Duke Children's Miracle Network Research Grant, American Liver Foundation/American Association of the Study of Liver Diseases Sheila Sherlock Translational Research Award

References

1. Patton HM, Patel K, Behling C, et al. The impact of steatosis on disease progression and early and sustained treatment response in chronic hepatitis C patients. *J Hepatol* 2004;40:484–90. [PubMed: 15123364]
2. Rubbia-Brandt L, Quadri R, Abid K, et al. Hepatocyte steatosis is a cytopathic effect of hepatitis C virus genotype 3. *J Hepatol* 2000;33:106–15. [PubMed: 10905593]

3. Jhaveri R, McHutchison J, Patel K, Qiang G, Diehl AM. Specific polymorphisms in hepatitis C virus genotype 3 core protein associated with intracellular lipid accumulation. *J Infect Dis* 2008;197:283–91. [PubMed: 18177246]
4. Miyanari Y, Atsuzawa K, Usuda N, et al. The lipid droplet is an important organelle for hepatitis C virus production. *Nat Cell Biol* 2007;9:1089–97. [PubMed: 17721513]
5. Gastaminza P, Cheng G, Wieland S, Zhong J, Liao W, Chisari FV. Cellular determinants of hepatitis C virus assembly, maturation, degradation, and secretion. *J Virol* 2008;82:2120–9. [PubMed: 18077707]
6. Boulant S, Targett-Adams P, McLauchlan J. Disrupting the association of hepatitis C virus core protein with lipid droplets correlates with a loss in production of infectious virus. *J Gen Virol* 2007;88:2204–13. [PubMed: 17622624]
7. Shavinskaya A, Boulant S, Penin F, McLauchlan J, Bartenschlager R. The lipid droplet binding domain of hepatitis C virus core protein is a major determinant for efficient virus assembly. *J Biol Chem* 2007;282:37158–69. [PubMed: 17942391]
8. Boulant S, Montserret R, Hope G, et al. Structural determinants that target the hepatitis C virus core protein to lipid droplets. *J Biol Chem*. 2006
9. Hope RG, Murphy DJ, McLauchlan J. The domains required to direct core proteins of hepatitis C virus and GB virus-B to lipid droplets share common features with plant oleosin proteins. *J Biol Chem* 2002;277:4261–70. [PubMed: 11706032]
10. Abid K, Pazienza V, de Gottardi A, et al. An in vitro model of hepatitis C virus genotype 3a-associated triglycerides accumulation. *J Hepatol* 2005;42:744–51. [PubMed: 15826725]
11. Greenwel P, Rubin J, Schwartz M, Hertzberg EL, Rojkind M. Liver fat-storing cell clones obtained from a CCl₄-cirrhotic rat are heterogeneous with regard to proliferation, expression of extracellular matrix components, interleukin-6, and connexin 43. *Lab Invest* 1993;69:210–6. [PubMed: 8394478]
12. Yang L, Jhaveri R, Huang J, Qi Y, Diehl AM. Endoplasmic reticulum stress, hepatocyte CD1d and NKT cell abnormalities in murine fatty livers. *Lab Invest* 2007;87:927–37. [PubMed: 17607300]
13. Moriya K, Yotsuyanagi H, Shintani Y, et al. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. *J Gen Virol* 1997;78(Pt 7):1527–31. [PubMed: 9225025]
14. Wakita T, Pietschmann T, Kato T, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 2005;11:791–6. [PubMed: 15951748]
15. Gottwein JM, Scheel TK, Hoegh AM, et al. Robust hepatitis C genotype 3a cell culture releasing adapted intergenotypic 3a/2a (S52/JFH1) viruses. *Gastroenterology* 2007;133:1614–26. [PubMed: 17983807]

Abbreviations

HCV	Hepatitis C virus
GFP	-green fluorescent protein
ORO	Oil Red O stain
IF	Immunofluorescence

Figure 1. Recombinant adenovirus infection of 5H cultured cells

Cells were infected with recombinant adenoviruses expressing green fluorescent protein (GFP) or HCV Core protein (HCV). Cells expressing GFP after infection showed sparse small lipid droplets when stained with Oil Red O (ORO) compared to cells expressing HCV Core which demonstrated large collections of lipid droplets. Note that HCV Core protein co-localized perfectly with lipid droplets.

B)

HCV vs. GFP: $p < 0.0001$

Figure 2.

a- Primary hepatocytes infected with recombinant adenoviruses. Primary mouse hepatocytes were infected with the same GFP and HCV Core expressing adenoviruses. Cells expressing GFP have some lipid droplets that are small in size. This is in sharp contrast to cells expressing HCV Core protein which show numerous large lipid droplets on ORO staining.

Figure 2b- Quantitative analysis of lipid accumulation. Using MetaMorph software, % ORO stain per region in 20 high power fields were calculated for GFP and HCV Core expressing cells. HCV Core expressing cells had a much higher percentage of ORO per image than GFP expressing cells (40% vs. 12%, $p < 0.0001$).

Figure 3. HCV Core co-localization with lipid droplets

Primary mouse hepatocytes were infected with HCV Core expressing adenoviruses. Note the co-localization of Core with the lipid droplets as indicated by the yellow arrows in both the IF and ORO images.