Autophagy Protects Cells From HCV-Induced Defects in Lipid Metabolism

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BACKGROUND & AIMS: Autophagy is a lysosome-mediated catabolic process that mediates degradation and recycling of all major components of eukaryotic cells. Different stresses, including viral and bacterial infection, induce autophagy, which can promote cell survival by removing the stress inducer or by attenuating its dangerous effects. High levels of autophagy occur during infection of cells with hepatitis C virus (HCV), but the clinical relevance of this process is not clear. **METHODS:** Levels of autophagy were analyzed in liver biopsy samples from 22 patients with HCV infection using microtubule-associated protein-1 light chain 3 immunoblotting; associations with histological and metabolic parameters were evaluated by Pearson correlation analysis. We investigated the role of HCV-induced autophagy in lipid degradation in cells infected with the virus or replicons, and analyzed autophagosome contents by confocal microscopy and by measuring lipid levels after inhibition of autophagy by Beclin 1 knockdown or lysosome inhibitors. **RESULTS:** In liver biopsy samples from patients with HCV, there was an inverse correlation between microvesicular steatosis and level of autophagy ($r = -0.617$; $P = .002$). HCV selectively induced autophagy of lipids in virus-infected and replicon cells. In each system, autophagosomes frequently colocalized with lipid deposits, mainly formed by unesterified cholesterol. Inhibition of the autophagic process in these cells significantly increased the induction of cholesterol accumulation by HCV. **CONCLUSIONS: Autophagy counteracts the alterations in lipid metabolism induced by HCV. Disruption of the autophagic process might contribute to development of steatosis in patients with HCV.**

Keywords: Liver Disease; NASH; Lipid Accumulation; Lipoprotein.

BASIC AND TRANSLATIONAL LIVER

Abnormal accumulation of fatty deposits in the liver, or steatosis, is a common feature of hepatitis C virus (HCV) infection, occurring in about 50% or more of patients.[1] Several studies point to steatosis as an important cofactor for fibrosis and disease progression in chronic hepatitis C.[2–7] Steatosis is significantly more severe in patients infected with HCV genotype 3 than with other genotypes, this being associated with specific polymorphisms of the HCV Core protein.[2,8,9] Various metabolic parameters are also associated with onset and severity of steatosis, regardless of HCV genotype, including body mass index, hyperlipidemia, type 2 diabetes, and insulin resistance.[10–13]

Many functional interactions between HCV and host cell lipid metabolism have been characterized.[14] HCV infection is associated with enhanced lipogenesis, reduced β-oxidation of lipids, and decreased lipoprotein secretion.[14,15] HCV modulates lipid homeostasis by increasing the activity of sterol regulatory element binding proteins and peroxisome proliferator–activated receptor-γ, 2 key regulators of de novo lipid biosynthesis.[16,17] On the other hand, HCV-infected patients with steatosis show reduced messenger RNA levels of peroxisome proliferator−activated receptor-α, a factor involved in β-oxidation and transport of fatty acids[18] when compared to those without steatosis.[19] Lipoprotein assembly and secretion is also altered by HCV through the interaction of HCV Core protein with the microsomal triglyceride transfer protein.[20] In addition, HCV Core and nonstructural protein 5A proteins have been found to be associated with different apolipoproteins involved in triglyceride storage and secretion.[21–24] In line with these findings, HCV patients exhibit low levels of circulating cholesterol compared with uninfected controls, with an additional decrease observed in HCV patients displaying steatosis.[25]

It is well established that HCV exploits lipid metabolism to accomplish several steps of its life cycle. HCV associates with lipoproteins to circulate in the bloodstream, to enter into and to be released from target cells.[14,15,26–28] HCV RNA replication is strictly dependent on host cell lipid metabolism, with a crucial role played by intermediates of cholesterol synthesis.[29–32] In addition, the association of HCV Core protein and HCV replication complex to lipid droplets is critical for virus assembly and release.[33]

Autophagy is an evolutionary-conserved cellular process that mediates the degradation of bulk cytoplasm and entire organelles.[34,35] In this process, double-membrane vesicles, termed *autophagosomes*, wrap around portions of cytosol and transport them to the lysosome for degradation.[36] Au-

Abbreviations used in this paper: GFP, green fluorescent protein; HCV, hepatitis C virus; LC3, microtubule-associated protein-1 light chain 3; NAFLD, nonalcoholic fatty liver disease.

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tophagy has generally been considered to be a nonselective process aimed at recycles intracellular constituents to provide an alternative source of substrates for de novo biosynthesis and energy production.[34] However, it is now evident that selective forms of autophagy are responsible for the specific removal of aggregate-prone proteins, damaged or supernumerary organelles, and microbes.[37,38] In addition, the autophagic process has recently been shown to be involved in the mobilization of intracellular lipid stores.[39] The interplay between autophagy and lipid metabolism is bidirectional, as changes in the intracellular lipid content also modulate autophagic activity.[40]

High levels of autophagy in HCV-infected cells in vitro have been reported recently[41–47]; however, the relevance of autophagy in HCV patients remains less characterized. Here we show that autophagy levels inversely correlate with microvesicular steatosis in the liver of HCV patients. We also report on evidence that autophagy counteracts HCV-induced lipid accumulation in in vitro cell culture systems.

Materials and Methods

Cell Culture

HuH7 cells were purchased from the American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. Blasticidin-resistant replicon cells are a mixed population of HuH7 cells transfected with the HCV genotype 1b (Con1) subgenomic replicon carrying a blasticidine resistance gene.[26] Rep Neo is a clonal cell line (previously described as Rep60) harboring the HCV genotype 1b (Con1) subgenomic replicon carrying a neomycin resistance gene.[26,48] The HCV cell culture infection system, HCVcc, based on the HCV JFH-1 molecular clone, has been described previously.[49] Huh7.5.1 and Huh7.5.1 cherry-microtubule–associated protein-1 light chain 3 (LC3) cells were infected with JFH1 virus (multiplicity of infection = 0.1) and harvested 5 days post infection. Recombinant human interferon alfa (PeproTech, Rocky Hill, NJ) was used as described.[48] For autophagy induction, cells were cultured for 4 hours in Earle's balanced salt solution (Sigma-Aldrich). To inhibit cholesterol synthesis, cells were incubated in complete medium in the presence of 10 μM Mevastatin (Sigma-Aldrich) for 36 h before the assay. To block lysosomal activity, cells were incubated in complete medium in the presence of 5 nM Bafilomycin A1 (Sigma-Aldrich) for 4 h before the assay.

Study Population

A retrospective review of medical records of HCV patients attending one of the outpatient units of the INMI "Lazzaro Spallanzani" in Rome between January 2004 and December 2007 was performed to select patient biopsies analyzed in this study. HCV patients were characterized by the presence of positive HCV RNA by qualitative polymerase chain reaction (COBAS AMPLICOR HCV Test, version 2.0; Roche Molecular Systems Inc., Pleasanton, CA; limit of detection: 50 IU/mL), with persistently abnormal alanine aminotransferase and liver histology of chronic hepatitis (eg, any degree of fibrosis, including cirrhosis), and by alcohol consumption <20 g/day at least in the last year.

Exclusion criteria were (1) advanced cirrhosis (Child–Pugh B and C); (2) hepatocellular carcinoma; (3) other causes of liver disease or mixed etiologies (eg, excessive alcohol consumption, hepatitis B, autoimmune liver disease, Wilson disease, hemochromatosis, α1-antitrypsin deficiency); (4) human immunodeficiency virus infection; (5) previous treatment with antiviral therapy, immunosuppressive drug, and/or regular use of steatosis-inducing drugs evaluated by a questionnaire (eg, corticosteroid, valproic acid, tamoxifen, amiodarone); (6) previous diagnosis of diabetes mellitus type 1 or type 2, and/or fasting blood glucose ≥126 mg/dL, and/or current therapy with insulin or oral hypoglycemic agents; and (7) active intravenous drug addiction or use of cannabis.

Genotyping was performed by INNO-LiPA HCV II (Bayer HealthCare, Berkeley, CA). Liver biopsies, performed percutaneously with a Menghini needle, were assessed histologically for fibrosis (Masson trichrome) and activity (H&E) according to the Metavir score and for steatosis. Hepatocytes that contained 1 large vacuole of fat that displaced the nuclei to the periphery of the cell were considered to have macrovesicular steatosis. When the cytoplasm contained many small fatty inclusions and the nuclei remained in the center of the cell, steatosis was classified as microvesicular. Zonal steatosis refers to lipid deposits that occupied contiguous areas of the biopsy in discrete distributions rather than being scattered randomly on an individual cell basis. Other clinical parameters, described in Supplementary Table 1, were determined using standard diagnostic analysis.

We also studied patients with a diagnosis of nonalcoholic fatty liver disease (NAFLD) based on the absence of alcohol intake, the presence of biopsy-proven steatosis with/without necroinflammation and/or fibrosis, and a negative anti-HCV serum test. In addition, patients from whom a liver biopsy was taken during programmed laparoscopic cholecystectomy were also studied. All of them had histologically normal liver; normal fasting glucose, cholesterol and triglycerides; normal serum aminotransferase levels; and no evidence of viral infections (eg, hepatitis B virus, HCV, and human immunodeficiency virus). In addition, none of these patients drank alcohol or used potentially hepatotoxic drugs. The Institutional Human Ethics Committee approved the study procedures, and written informed consent was obtained from all patients before liver biopsy.

Liver biopsies used in the analysis of the expression of LC3 were frozen within 1 h after collection and stored at −80°C. A sample of the biopsy was placed in formalin for a histological evaluation. Liver biopsies used for immunoblottings were homogenized in CelLytic Mammalian Tissue extraction reagent (Sigma-Aldrich).

Statistical Analysis

Statistical analyses were performed using the StatistiXL package (University of Western Australia, version 1.8, released on December 31, 2007) as an add-in to Windows. Probabilities <.05 were considered significant and reported P values are 2-sided. The Pearson correlation coefficient (R) was used to assess the correlation between clinical parameters and the expression of LC3 in liver biopsies from patients included in the study.

Results

Autophagy Levels Inversely Correlate With Microvesicular Steatosis in HCV Patients

In order to investigate the relevance of autophagy in HCV infection in vivo, autophagy levels were assessed in liver biopsies of 22 HCV patients by immunoblotting analysis of the autophagic marker LC3 and quantified as