Knockdown of Autophagy Enhances the Innate Immune Response in Hepatitis C Virus–Infected Hepatocytes

Shubham Shrivastava,1 Amit Raychoudhuri,1 Robert Steele,1 Ranjit Ray,2 and Ratna B. Ray1,2

The role of autophagy in disease pathogenesis following viral infection is beginning to be elucidated. We have previously reported that hepatitis C virus (HCV) infection in hepatocytes induces autophagy. However, the biological significance of HCV-induced autophagy has not been clarified. Autophagy has recently been identified as a novel component of the innate immune system against viral infection. In this study, we found that knockdown of autophagy-related protein beclin 1 (BCN1) or autophagy-related protein 7 (ATG7) in immortalized human hepatocytes (IHHs) inhibited HCV growth. BCN1- or ATG7-knockdown IHHs, when they were infected with HCV, exhibited increased expression of interferon-β, 2′,5′-oligoadenylate synthetase 1, interferon-α, and interferon-α-inducible protein 27 messenger RNAs of the interferon signaling pathways in comparison with infected control IHHs. A subsequent study demonstrated that HCV infection in autophagy-impaired IHHs displayed caspase activation, poly(adenosine diphosphate ribose) polymerase cleavage, and apoptotic cell death.

Conclusion: The disruption of autophagy machinery in HCV-infected hepatocytes activates the interferon signaling pathway and induces apoptosis. Together, these results suggest that HCV-induced autophagy impairs the innate immune response. (Hepatology 2011;53:406-414)

Hepatitis C virus (HCV) infection affects nearly 3.3 million people and is the most common cause of cirrhosis and hepatocellular carcinoma in the United States.1 The currently approved therapy for the treatment of HCV is pegylated interferon in combination with ribavirin.2,3 Although several advances have shown promise in improving the management of HCV infection, nevertheless, it remains a major health problem.4-6 HCV is a member of the Flaviviridae family, and its genome contains a positive-strand RNA approximately 9.6 kb long. The HCV genome encodes a polyprotein precursor of approximately 3000 amino acids that is cleaved by both viral and host proteases into structural (core, E1, E2, and p7) and nonstructural proteins [nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B]. HCV-infected cells accumulate lipid droplets and play an important role in the assembly of virus particles.7-9

Autophagy is a catabolic process by which cells remove their own damaged organelles and long-lived proteins for the maintenance of cellular homeostasis. During autophagy, the double-membrane vesicles engulf the damaged organelles and eventually fuse with the lysosomes for degradation. Autophagy is up-regulated in response to extracellular or intracellular stress and signals, such as starvation, growth factor deprivation, endoplasmic reticulum stress, and pathogen infection.10,11 Viruses are obligate intracellular parasites, and their survival is linked to their ability to subvert cellular antiviral defenses and to regulate cellular
processes necessary for their own replication. We have shown that HCV genotype 1a or genotype 2a infection induces autophagy in immortalized human hepatocytes (IHHs). Autophagy induction was subsequently reported with an HCV genotype 1b or 2a subgenomic replicon or infection with HCV genotype 2a (clone JFH1) in Hu7.5 cells. Although HCV-induced autophagy was established, whether autophagy helps in host cell survival or is beneficial for HCV multiplication remains unknown. Some viruses, such as cytomegalovirus, Kaposi’s sarcoma–associated herpes virus, and human herpes simplex virus 1, have evolved strategies to suppress autophagy for their own survival. In herpes simplex virus infection, infected cell protein 34.5 suppresses autophagy by binding to beclin 1 (BCN1) and blocks the initiation of autophagy. Certain viruses, such as mouse hepatitis virus, poliovirus, coxsackievirus, and dengue virus, exploit the elements of the autophagy system for their own replication. In mammalian systems, BCN1 recruits other autophagy proteins to initiate the formation of the pre-autophagosomal membrane. Autophagy-related protein 7 (ATG7) is required in conjunction with ATG12 and ATG5 to form autophagosomes. We have reported previously that HCV infection induces BCN1 expression. In this study, we have demonstrated that HCV infection in autophagy-knockdown cells activates the interferon (IFN) signaling pathway and apoptosis.

Materials and Methods

Cell Culture, Transfection, and HCV Infection. IHHs and human hepatoma (Hu7.5) cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 U/mL penicillin G, and 100 μg/mL streptomycin at 37°C in a 5% carbon dioxide atmosphere. We grew HCV genotypes 1a (clone H77) and 2a (clone JFH1) in IHHs as previously described. HCV genotype 2a was initially grown in Hu7.5 cells in this study. For infection, IHHs (3 × 10^5 cells) were incubated with HCV genotype 1a (clone H77, 5.3 × 10^8 IU) and HCV genotype 2a (clone JFH1, 1.2 × 10^8 IU) in a minimum volume of the medium. After 8 hours of virus adsorption on hepatocytes, Dulbecco’s modified Eagle’s medium, supplemented with 5% heat-inactivated fetal bovine serum, was added. IHHs, transfected with control (scrambled) or BCN1 small interfering RNA (siRNA) or ATG7 siRNA with Lipofectamine 2000 (Invitrogen, Carlsbad, CA), were similarly infected with cell culture–grown HCV. The siRNAs used in this study were mixtures of three siRNAs and were purchased from Santa Cruz (the sequences are not disclosed). The HCV titer was measured as previously described. Briefly, HCV infectivity from a 3-day culture supernatant was titrated by an endpoint dilution assay in a 96-well plate. Virus inocula were serially diluted and used to infect six replicate wells of naive IHHs growing in a microtiter plate. Three days post-infection, the cells were washed, fixed with cold methanol, and incubated with a mouse monoclonal NS5A-specific antibody (HL1126, which was kindly provided by Chen Liu) at 4°C overnight. After they were washed with phosphate-buffered saline (PBS), the cells were incubated with anti-mouse immunoglobulin conjugated with Alexa 488 (Invitrogen) for 60 minutes at room temperature and were visualized under a fluorescence microscope.

Microtubule-associated protein 1 light chain 3 (LC3), a homologue of Apg8p essential for autophagy in yeast, is associated with autophagosome membranes after processing and is indispensable for the elongation of autophagic vesicles. Two forms of LC3, LC3-I and LC3-II, are produced posttranslationally in various cells. LC3-I is cytosolic, whereas LC3-II is membrane-bound. Lipidated LC3 (LC3-II) is a useful marker of autophagic membranes, and autophagosomes are visualized as bright green fluorescent protein (GFP)–LC3 puncta by fluorescence microscopy. As a readout of autophagy induction, cells were transfected with GFP-LC3 as described previously. Cells containing three or more GFP-LC3 dots were defined as autophagy-positive cells. For LysoTracker Red staining, the cells were treated with 1 μM LysoTracker Red DND-99 (Invitrogen) at 37°C for 30 minutes. Control IHHs and BCN1-knockdown IHHs (siBCN1 IHHs) or ATG7-knockdown IHHs (siATG7 IHHs) were starved in Hank’s balanced salt solution (Lonza) for 120 minutes in the presence of LysoTracker Red dye. Cells then were fixed in 3.7% formaldehyde, and nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen). Three-channel optical images (DAPI, GFP, and LysoTracker Red) were collected with the sequential scanning mode (405-, 488-, and 543-nm excitation, respectively, and 450-, 522-, and 595-nm emission, respectively) of the Olympus FV1000 confocal system.

RNA Quantitation. Control IHHs, siBCN1 IHHs, or siATG7 IHHs were infected with HCV and incubated for 72 hours. Total RNA was isolated with the Qiagen RNeasy kit (Qiagen, Valencia, CA). Complementary DNA was synthesized with a random hexamer and the Superscript III reverse-transcriptase kit.
Real-time polymerase chain reaction (PCR) was performed with complementary DNA for RNA quantitation with the TaqMan gene expression PCR master mix (Applied Biosystems) and 6-carboxy fluorescein (FAM) labeled reporter minor groove binding (MGB) FAM-MGB probes for interferon-α-inducible protein 27 (IFI27; Hs00271467_m1), IFN-β (Hs02621180_s1), 2′,5′-oligoadenylate synthetase 1 (OAS1; Hs00973637_m1), and IFNA1 (Hs00353738_s1). The FAM-MGB probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Hs99999905_m1) was used as an endogenous control.

Cell Death Assay. HCV-infected control IHHs, siBCN1 IHHs, or siATG7 IHHs were assayed for cell viability/death after 72 hours of infection with the Live/Dead two-color fluorescence assay according to the manufacturer’s instructions (Molecular Probes, Carlsbad, CA). Infected IHHs were washed in PBS and exposed to 4 μM calcein AM and 2 μM ethidium homodimer in PBS for 30 minutes at room temperature. Dye uptake was detected for fluorescein (for calcein in live cells) and Texas red (for ethidium homodimer in dead cells). Image analysis for quantifying live and dead cells was performed with ImageJ software.

Immunoblot Analysis. HCV-infected IHHs, siBCN1 IHHs, or siATG7 IHHs were lysed with a sodium dodecyl sulfate sample buffer. Proteins were subjected to electrophoresis on polyacrylamide gel and were transferred onto a nitrocellulose membrane. The membrane was probed with an antibody for poly(adenosine diphosphate ribose) polymerase (PARP), caspase-9, or caspase-3 (Cell Signaling Technology, Beverly, MA). Proteins were detected with an enhanced chemiluminescence western blot substrate (Pierce, Rockford, IL). The membrane was reprobed for tubulin as an internal control protein.

Results

Knockdown of BCN1 Reduces HCV Growth. BCN1 is one of the upstream molecules that recruit other autophagy proteins to initiate the autophagy signaling pathway.21,22 BCN1 forms a complex with Vps34 (vacuolar protein sorting 34), phosphoinositide 3-kinase, p150 and ATG14-like protein and promotes autophagic vesicle formation.22 We chose to use BCN1 in examining whether knockdown of the autophagy gene altered HCV growth. IHHs were transfected with control (scrambled) or BCN1 siRNA, and the expression of BCN1 was examined at the messenger RNA (mRNA) and protein levels. A significant inhibition of BCN1 at the RNA (~6-fold) and protein levels (>90%) was observed after the siRNA treatment (Fig. 1A). IHHs treated with ATG7 siRNA did not display knockdown of BCN1 expression, and this suggested the specificity of BCN1 siRNA. We did not observe a difference in cell viability in BCN1-knockdown cells versus control IHHs. To examine the induction of autophagy in control IHHs or siBCN1 IHHs, cells were starved with nutrients. The presence of autolysosomes in cells was assessed with staining by GFP-LC3 and LysoTracker Red, which stains for acidic organelles such as lysosomes. Clearly, in starved IHHs, LC3 was colocalized with LysoTracker Red, and this suggested the formation of the autolysosomes (Fig. 1B). As expected, autolysosome formation was not observed in starved siBCN1 IHHs or uninfected IHHs, as shown by the distribution of LC3.
HCV-infected control IHHs and siBCN1 IHHs were transfected with LC3-GFP to examine the induction of autophagy. As expected, LC3 redistribution into discrete dots was markedly increased in HCV-infected IHHs versus HCV-infected siBCN1 IHHs (Fig. 2A). HCV-infected IHHs displayed LC3-II expression, whereas a predominant LC3-I band was observed in HCV-infected siBCN1 IHHs (data not shown). Next, control IHHs and siBCN1 IHHs were infected with cell culture–grown HCV genotype 1a or 2a (H77 or JFH1 clone) for 3 days. We determined the effect of BCN1 knockdown on the release of infectious virus particles. The HCV titer in BCN1-knockdown infected hepatocytes was reduced in comparison with HCV-infected control IHHs (Fig. 2B). Therefore, the impairment of autophagy machinery reduces HCV production in IHHs.

**HCV Infection Enhances IFN Signaling in Autophagy-Knockdown IHHs.** We have seen earlier that HCV-infected IHHs display inhibition of IFN-α and IFI27 expression. Here, we asked whether the knockdown of the autophagy protein after HCV infection modulates the IFN signaling pathway. For this, the expression levels of IFN-β, OAS1, IFN-α, and IFI27 from control IHHs and siBCN1 IHHs were initially examined to verify that BCN1 knockdown has an effect on the IFN signaling pathway. A significant difference in the expression of these genes in control IHHs and siBCN1 IHHs was not observed (data not shown). We next examined the status of a number of IFN-stimulated genes in HCV-infected control IHHs and siBCN1 IHHs. We showed earlier that HCV infection in IHHs up-regulates IFN-β and OAS1. Here, the mRNA level of IFN-β and OAS1 was 6- to 10-fold higher in siBCN1 IHHs infected with HCV genotype 1a or 2a versus virus-infected control IHHs (Fig. 3). Because HCV infection of IHHs inhibits IFN-α and IFI27 mRNA expression, we wanted to examine whether IFN-α synthesis is altered in HCV-infected siBCN1 IHHs. The results displayed a significant increase in IFN-α and its downstream molecule IFI27 expression in HCV-infected siBCN1 IHHs versus HCV-infected control IHHs (Fig. 3). Together, these results indicate that HCV-induced autophagy suppresses the expression of IFN-stimulated genes.

To further confirm the modulation of IFN-stimulated genes in HCV-infected autophagy-knockdown cells, we used another autophagy protein, ATG7. Knockdown of ATG7 in IHHs (siATG7 IHHs) did not alter the cell viability or display any off-target effect (data not shown). Control IHHs or siATG7 IHHs were infected with HCV (genotype 1a) for 72 hours. Virus release was measured, and a decrease in HCV growth in siATG7 IHHs versus HCV-infected control IHHs was observed (Fig. 4A). OAS1 (Fig. 4B), IFNA1 (Fig. 4C), and IFI27 mRNA expression (Fig. 4D) was up-regulated in HCV-infected siATG7 IHHs versus HCV-infected control IHHs (Fig. 4). Similar results were obtained when cells were infected with HCV genotype 2a. Together, these results suggest that disruption of autophagy machinery induces the IFN-signaling pathway in HCV-infected hepatocytes.

**HCV Infection in Autophagy-Knockdown IHHs Induces Apoptosis.** We hypothesized that autophagy promotes the survival of HCV-infected cells for virus persistence. Therefore, the viability of HCV-infected control IHHs and siBCN1 IHHs was examined. For this, cells were initially stained with calcein AM and ethidium homodimer 1 dye to quantitate live and dead cells (Fig. 5A). The percentage of dead cells (red color) was significantly higher in HCV-infected siBCN1 IHHs (~70%) versus HCV-infected control IHHs (~20%). A time-course experiment involving cell viability after HCV infection was performed. A significant inhibition of cell viability was noted in HCV-infected siBCN1 IHHs versus control IHHs (Fig. 5B).
Subsequently, apoptosis as a possible mechanism of cell death was examined. HCV-infected control IHHs and siBCN1 IHHs were incubated for 72 hours. Cell lysates were examined for the induction of apoptosis. PARP was significantly cleaved to an 86-kDa signature peptide in HCV-infected siBCN1 IHHs in comparison with HCV-infected control IHHs (Fig. 5C). Our results also demonstrated that BCN1-knockdown IHHs infected with HCV induced caspase-9 and caspase-3 activation. Procaspase-9 and procaspase-3 were cleaved to 37- and 17-kDa protein bands, respectively (Fig. 5C). Similar results were obtained in HCV-infected siATG7 IHHs (data not shown). Therefore, it is conceivable that autophagy machinery is needed for HCV-infected cell survival, and impairment of this pathway induces apoptosis.
Discussion

Autophagy has recently been identified as a novel component of the innate immune system against viral infection. In this study, we have observed that HCV-infected siBCN1 IHHs do not induce autophagy, and virus growth is reduced. We have further demonstrated that HCV-infected siBCN1 IHHs induce IFN-β, OAS1, IFN-α, and IFI27 mRNA expression and apoptotic cell death. Similar results have also been obtained for HCV-infected ATG7-knockdown IHHs. We propose that HCV induces autophagy in favor of its own survival; inhibition of autophagic proteins enhances cell death; and as a result, virus growth is reduced (Fig. 6). This may have potential for future therapeutic modalities.

Autophagy plays a key role in recognizing signatures of viral infection and represents a critical effector mechanism for restricting virus production. Upon invasion by a pathogen, the host may initiate autophagosome formation as a cellular defense. Autophagy is proposed to serve as a scaffold for intracellular membrane–associated replication for RNA viruses. In rotavirus-infected cells, the NSP4 protein is involved in virus replication and colocalized with LC3 in a double-layered vesicular compartment, a site for nascent viral RNA replication. In dengue virus–infected cells, LC3 colocalizes with double-stranded RNA and with the NS1 protein; this suggests the presence of replication complexes in autophagic vesicles. We and others have shown that HCV induces autophagy through an accumulation of autophagosomes in infected hepatocytes without colocalization of HCV and autophagy-related proteins. Knockdown of autophagy proteins in HCV-infected cells or replicon-bearing hepatocytes does not impair the expression levels of mRNA or proteins. Insufficient packaging of viral RNA or a blockage of virus release may be a mechanism for suppression of HCV production in autophagy-impaired cells. Indeed, further work is necessary to understand the in-depth mechanism for suppression of infectious virus particle production. The cell type specificity is associated with autophagy machinery. For example, in lung epithelial A549 cells, autophagy machinery favors viral protein accumulation and an infectious viral yield, whereas autophagy has no effect on influenza A virus replication and viral titers in mouse embryo fibroblasts. In agreement with the previous reports of the HCV genotype 2a system in the Huh7 cell line or its derivatives, we also observed a reduction of infectious HCV particle release in autophagy-deficient IHHs.

ATG5 has been shown to be essential for the production of type I IFN in plasmacytoid dendritic cells infected with vesicular stomatitis virus by a mechanism presumed to involve the autophagy-mediated delivery of viral genetic material to endosomal toll-like receptors. On the other hand, several studies have shown that the absence or knockdown of autophagy genes in certain cell types can result in enhanced production of type I IFN or other cytokines, including proinflammatory molecules. In agreement with the latter, we have seen that HCV infection in BCN1- or ATG7-knockdown IHHs increases IFN-β, OAS1, and IFN-α...
synthesis and enhances IFI27 mRNA. The Atg5-Atg12 conjugate interacts between the caspase recruitment domains (CARDs) of retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (Mda5), and their adaptor protein (interferon beta promoter stimulator 1/mitochondrial antiviral signaling protein) to suppress the activity of such helicases in stimulating the production of type I IFN.32 HCV infection also cleaves these helicases and interferes with the IFN signaling pathway.35,36 Knockdown of BCN1 in IHHs does not induce IFN-related gene expression, and BCN1-knockdown cells infected with HCV do not induce autophagy. Therefore, it is possible that the autophagic machinery as well as HCV infection may suppress innate immune signaling by directly inhibiting the interactions with these helicases and their adaptor proteins. Thus, the autophagic machinery may serve a dual function in innate immune signaling by acting not only to modulate antiviral type I IFN responses in host cells but also to ensure homeostatic balance by preventing excess innate immune activation in other cell types.

Autophagy is also involved in biological pathways and possesses a dual role in mediating cell survival and cell death. Autophagy acts as a cell survival mechanism in tobacco mosaic virus: it restricts the virus to spreading from infected tissue to healthy tissue and regulates the programmed cell death in neighboring uninfected cells.37 In autophagy protein–deficient cells, influenza A virus induces apoptosis, and this suggests that autophagy favors the survival of infected cells.30 We have also documented an increase in apoptotic cell death in HCV-infected, autophagy-impaired IHHs, and this reflects that autophagy promotes cell survival. It is possible that HCV-mediated autophagy may sustain cell viability during virus-induced stress in order to prevent apoptosis. IFN-α can induce cell death by

![Fig. 5. HCV infection in autophagy-knockdown IHHs induces apoptotic cell death. (A) Control IHHs or siBCN1 IHHs infected with HCV were stained with calcein AM (green for live cells) and ethidium homodimer 1 dye (red for dead cells) to quantitate the live and dead cells by fluorescence microscopy. (B) Cell viability was examined in HCV-infected control IHHs or siBCN1 IHHs. The number of viable cells was counted at 1, 2, and 3 days by trypan blue exclusion. The results are presented as the means of three separate experiments with standard errors (P < 0.001). (C) HCV-infected control IHHs or siBCN1 IHHs were subjected to western blot analysis with a specific antibody for the detection of PARP, caspase-9, or caspase-3. PARP was significantly cleaved to an 86-kDa signature peptide in HCV-infected siBCN1 IHHs in comparison with HCV-infected control IHHs. Caspase-9 and caspase-3 activation was also observed in HCV-infected siBCN1 IHHs. The blot was reprobed with an antibody to tubulin for comparison with an equal protein load.](image_url)

![Fig. 6. Role of autophagy as a proposed mechanism for the establishment of persistent HCV infection.](image_url)
modulating the Janus kinase/signal transducer and activator of transcription (STAT) or phosphoinositide 3-kinase/protein kinase B signaling pathway, STAT3 activation, or cytokine induction. Therefore, IFN induction in HCV-infected, autophagy-impaired cells may mediate apoptotic cell death. Autophagy has been suggested to extend the survival time of human parvovirus B19–infected erythroid cells during viral expansion. Hepatitis B virus–encoded transcriptional transactivator protein X up-regulates BCN1 expression and stimulates autophagy. We have shown previously that HCV infection enhances BCN1. Therefore, it is plausible that HCV induces autophagy to prolong cell survival. This process may help to initiate the development of liver disease progression, including hepatocellular carcinoma, through an as yet uncharacterized mechanism.

In conclusion, the results from this study reveal that HCV infection in autophagy-knockdown cells induces the IFN signaling pathway and enhances hepatocyte death. Therefore, autophagy serves as a pivotal entity that may help HCV in establishing persistent infection, reducing antiviral innate immunity, and promoting cell survival. The interaction of the virus with the autophagy machinery involves multiple pathways that have only just begun to be characterized. However, other mechanisms, either simultaneously or subsequently, may also be involved in the establishment of chronic HCV infection in humans.

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References