

Hepatitis B virus promotes autophagic degradation but not replication in autophagosome

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Summary

In this study, we investigate the relationship of hepatitis B virus (HBV) infection and autophagy. HepG2 cells and HepG2 cells infected with HBV (HepG2.2.15) were transfected with GFP-LC3 (green fluorescence protein conjugated with microtubule-associated protein 1 light chain 3) expression vector and autophagy status was then examined with confocal microscope. HepG2.2.15 cells were further treated with serum-free medium or 3-methyladenine (3-MA), and subjected to Hepatitis B core antigen (HBcAg), Hepatitis B surface antigen (HBsAg), or hepatitis B polymerase protein detection by immunohistochemistry. Localization of the GFP-LC3 and the HBV proteins was observed by confocal fluorescence microscope. The level of SQSTM1/p62 protein was also evaluated by Western blot analysis. In contrast to a diffuse distribution in HepG2 cells, GFP-LC3 formed distinct punctate dots, which were further enhanced by nutritional starvation, in HepG2.2.15 cells. The expression of hepatitis B polymerase and HBcAg, but not HBsAg, was positively correlated with the autophagic intensity. However, no co-localizations were observed between HBV proteins and autophagosomes. Suppression of autophagy reduced the expression of hepatitis B polymerase and HBcAg, but not HBsAg. Western blot showed that SQSTM1/p62 protein level was declined in HepG2.2.15 cells comparing HepG2 cells, and further reduced while upon serum starvation. In conclusion, HBV infection induces autophagic degradation and autophagy. Autophagy is critical for HBV replication. However HBV replication does not take place in autophagosomes.

Keywords: Hepatitis B virus, autophagy, virus replication, HepG2 cell

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most malignant cancers worldwide, ranking the 5th highest in morbidity and the 3rd highest in mortality among

various cancers (1). Chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection is found to be associated with nearly 80% of HCC (2). HCC is particularly troublesome in China since over 55% new HCC cases worldwide were discovered in China, strongly due to the presence of 93 million HBV carriers (3,4). However, the pathogenesis of HBV and its underlying mechanisms for carcinogenesis is not well understood. Thus, this lack of understanding hinders the effective therapeutic approaches for curing HBV infections or decreasing its contribution to carcinogenesis.

Autophagy is a conserved cellular process in which double-membraned vesicles are formed, containing excess or dysfunctional proteins as well as cellular

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organelles, and delivered to lysosomal machinery for degradation. It is a highly regulated process that is essential for maintaining cellular homeostasis in response to multiple stress signals, including nutrient starvation, growth factor deprivation, genotoxic stress, and hypoxia. Autophagy is also known as one of the host defense responses against infections. It has been shown that autophagy is involved in selectively targeting intracellular pathogens, leading to their direct elimination (5-9). It also plays a role in the MHC class II antigen presenting for foreign antigens (10). Thus, some bacteria and viruses developed strategies to suppress or bypass cellular autophagy to ensure their survival (11). Some viruses, such as poliovirus, rhinoviruses, mouse hepatitis virus, SARS-CoV, even hijacked the components of autophagic pathway to facilitate their own replication (12,13).

It has been demonstrated that HBV induces autophagy and uses it to facilitate its DNA replication. This process is mediated by HBV X (HBx) protein, which binds to and activates class III phosphatidylinositol-3-kinase (C3-P13K), an enzyme important for autophagy initiation (14). There is also evidence showing that HBx protein can up-regulate the expression of Beclin 1, the mammalian orthologue of the yeast autophagy protein Apg6 (15). However, the mechanism of how the autophagy contributes to viral DNA replication remains unclear.

In this study, we explored the role of autophagosomes in HBV replication by observation of autophagosomes and HBV with a confocal microscope. Meanwhile, SQSTM1/p62, one of the proteins involved in autophagy, was analyzed by western blotting for further understanding the function of autophagy.

2. Materials and Methods

2.1. Strain, plasmid, and cell lines

Competent *Escherichia coli* strain DH5 α was purchased from Transgen Biotech Corp. (Beijing, China). Expression plasmid pEGFP-C1-LC3 and the HepG2 cell line were kindly provided by Prof. Hongbing Zhang. The HepG2.2.15 cell line was obtained from Peking University Hepatology Institute, which was transfected with HBV genome and thus stably express HBV proteins and produce virions.

2.2. Cell culture and transfection

Both HepG2 and HepG2.2.15 cells were maintained in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal calf serum, 100 units/mL penicillin, and 100 mg/mL streptomycin. The HepG2.2.15 cultural medium has additions of G418 (Wako, Japan) for a final concentration of 380 μ g/mL. The pEGFP-C1-LC3 transfection was performed using Lipofectamine 2000 (Invitrogen, CA, USA).

2.3. Immunofluorescence

HepG2.2.15 cells plated on coverslips were washed with PBS and fixed with 4% (v/v) formaldehyde in PBS for 10 min at room temperature. Cells were then permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature followed by blocking in TBS-T solution (25 mM Tris, pH 7.4, 3.0 mM KCl, 140 mM NaCl and 0.05% Tween 20) containing 5% (w/v) BSA (FirstLink, UK) for 30 min. Cells were incubated with one of the primary antibodies, anti-HBcAg (Abcam, CA, USA), anti-HBsAg (Abcam), or anti-hepatitis B polymerase antibody (Santa Cruz Biotechnology, CA, USA) at 4°C overnight. Goat anti-mouse IgG antibody conjugated to Cy3 (Beyotime Institute of Biotech, China) was used as a secondary antibody. Post-staining images were taken with FLUOVIEW10-ASW laser scanning confocal microscope (Olympus, Japan).

2.4. Western blot analysis

Cell lysates were subjected to SDS/PAGE on a 4-15% gradient gel and transferred onto a polyvinylidene fluoride (PVDF) membrane. After blocking with 5% non-fat dry milk in Tris-buffered saline, the membrane was incubated with primary rabbit monoclonal antibody against P62 protein (Santa Cruz Biotechnology), followed by a secondary horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (Santa Cruz Biotechnology). Subsequently, the blot was developed using an ECL detection kit (Thermo, CA, USA).

3. Results and Discussion

3.1. HBV replication induces autophagic vacuole formation

Autophagy initiates with the formation of phagophore (an isolation membrane). One of the components involved in this early event is the protein LC3, which becomes lipidated (LC3-II) and associates with the newly formed double-membrane vesicles. It has been shown that exogenous GFP-LC3 (green fluorescence protein conjugated with microtubule-associated protein 1 light chain 3) behaves in a similar fashion as does endogenous LC3, upon autophagy induction. We took advantage of the GFP-LC3 fusion protein, and studied whether continual expression of HBV proteins leads to autophagy induction in HepG2 cells. We transfected the plasmid expressing GFP-LC3 into both HepG2 and HepG2.2.15 cells, and studied the distribution of the GFP signals in the cells through fluorescence microscopy. A diffused distribution of GFP signal was observed in HepG2 cells (Figure 1A) that was consistent with the distribution of the soluble form of LC3 in the absence of autophagy. The GFP signal in HepG2.2.15 cells, however, appeared as punctuated dots much

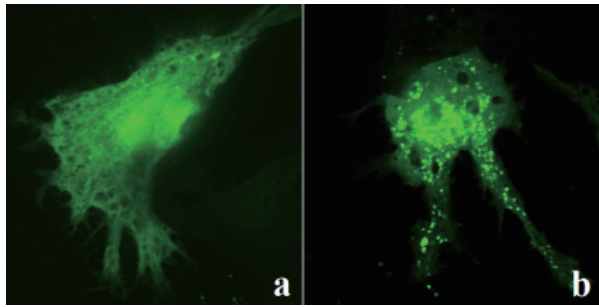


Figure 1. Induction of autophagic vacuoles by HBV. HepG2 cells (**panel a**) and HepG2.2.15 cells (**panel b**) were transfected with pEGFP-C1-LC3. Images were taken at 24 h post-transfection. The Green color was shown in a diffused pattern in HepG2 cells and in punctate dots in HepG2.2.15 cells. The punctate dots represent formation of autophagic vacuoles.

different than what was observed in HepG2 cells (Figure 1B). The punctuated localization is consistent with the observation that during autophagy the lipidated LC3 is associated with the autophagosomal membrane. The production of HBV by HepG2.2.15 cells was verified by FQ-PCR (data not shown). These observations indicated that the presence of HBV proteins induce autophagy in host cells.

This autophagy can be further augmented by nutrient starvation, one of the many common autophagy-inducing signals. HepG2.2.15 cells expressing GFP-LC3 were cultured in serum-free DMEM medium for 24 h. As shown in Figure 2, the intensity of green fluorescence produced from GFP-LC3 became stronger and more punctuated than those observed in cells cultured under normal conditions (comparing panels **a** and **d**). This indicated that nutrient starvation could further induce autophagy process in cells with an already elevated autophagy level due to virus expression.

HBV induced autophagy can be abolished by 3-methyladenine (3-MA), an inhibitor for type III PI3 kinase Vps34. When GFP-LC3 expressing HepG2.2.15 cells were treated with 10 mM 3-MA (Sigma, CA, USA), the distribution of green fluorescence became more diffused than that of the untreated control, suggesting localization of LC3 is more cytosolic (Figure 2 comparing panels **g** and **d**).

3.2. The expression of HBsAg, HBcAg, and hepatitis B polymerase and their localization

We then used immunofluorescence to study whether autophagy is required for the expression of HBV proteins, specifically that of hepatitis B polymerase, HBcAg, and HBsAg.

We did not find significant changes in the expression of hepatitis B polymerase or HBcAg when autophagy is further enhanced by starvation. The intensities in fluorescence reflecting the abundances of protein were not significantly different in cells growing in normal conditions or starved conditions (Figures 2A and 2B,

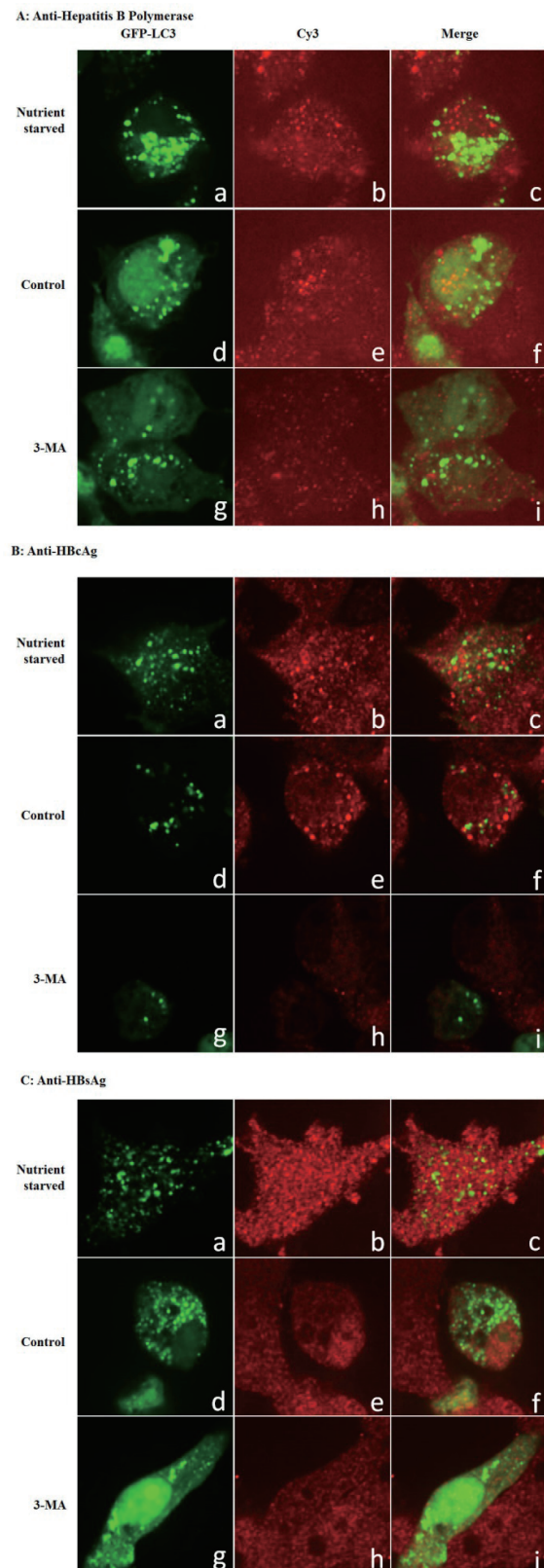


Figure 2. The effect of autophagy on HBV protein expressions. HepG2.2.15 cells transfected with pEGFP-C1-LC3 were cultured in serum-free DMEM medium, normal DMEM medium as control, or with 3-MA in DMEM medium. HBV protein expressions were detected with antibodies against hepatitis B polymerase (A), HBcAg (B) or HBsAg (C) and subsequent with the Cy3-conjugated goat anti-mouse secondary antibody. Localization of autophagic vacuoles and HBV was observed by merging panels between GFP-LC3 (green) and Cy3 (red) signals.

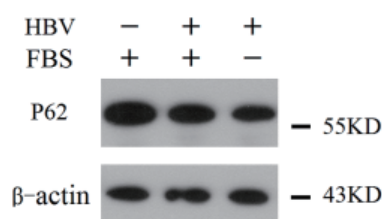


Figure 3. The autophagy induced by HBV leads autophagic degradation. HepG2 cells were cultured in normal DMEM medium (**lane 1**), while HepG2.2.15 cells were cultured either in normal DMEM medium (**lane 2**) or serum-free DMEM medium (**lane 3**). Cells were lysed for Western-blot analysis using the rabbit monoclonal antibody against P62 protein antibody (top panel). Actin (bottom panel) was also shown as a loading control.

comparing panels **b** and **e**). However, the abundances of both proteins reduced in cells where autophagy is inhibited by 3-MA (Figures 2A and 2B, comparing panels **e** and **h**). HBsAg expression seems to be different. We found that the fluorescence intensity of HBsAg is increased during starvation, when autophagy is enhanced in addition to the induction by HBV. Inhibition of autophagy by 3-MA has little effect on its expression (Figure 2C, comparing panel **e** and **h**).

It is reported that several human viruses, such as HCV, use autophagic vesicles as membranous support for translation of viral protein and enhancement of viral RNA replication (12,13,16). We investigated whether HBV similarly uses the autophagic vesicles as its location of replication. The merged images between GFP-LC3 and antibody staining for viral proteins, HBV polymerase, HBcAg, or HBsAg showed that the membrane-associated LC3 does not co-localize with viral proteins, suggesting that HBV may not occupy the autophagic vesicles as its location for viral protein translation and DNA replication (Figure 2, panels **c**, **f**, and **i**).

3.3. The autophagy induced by HBV leads autophagic degradation

SQSTM1/p62 protein is an ubiquitin-binding scaffold protein that binds directly to LC3 *via* the LC3 interacting region (LIR), and interacts with ubiquitinated proteins through the C-terminal ubiquitin associated (UBA) domains. SQSTM1/p62 can link ubiquitinated proteins to autophagosome for degradation, and itself can also be degraded through autophagy (17). The cellular levels of SQSTM1/p62 vary with the induction or inhibition of autophagy; it therefore often serves as a marker for autophagic degradation. We studied whether autophagy induced by HBV leads to autophagic degradation by detecting the SQSTM1/p62 protein level with Western blot. Analysis showed that SQSTM1/p62 protein level was decreased in HepG2.2.15 cells comparing to HepG2 cells, and it was further reduced in HepG2.2.15 cells cultured in serum-free DMEM medium (Figure 3).

This indicated that autophagy induced by HBV leads to autophagic degradation.

Autophagy is a protective process that recycles damaged organelles and unwanted proteins to ensure cell viability under starvation and stress conditions. Increasing amounts of evidence has shown that it is also involved in regulating innate and adaptive immunity. It is particularly important in the cellular defense against intracellular pathogens, in which xenophagy recognizes intracellular microbes and targets them to the autophagy pathway for degradation. Thus, many pathogens, including viruses, have developed strategies for self-protection and further manipulate or utilize autophagy to their own advantages. For example, Epstein-Barr virus (EBV) and Kaposi's Sarcoma-Associated Herpes Virus (KSHV) negatively regulate autophagy during latency, whereas poliovirus (18,19), porcine reproductive and respiratory syndrome virus (PRRSV) (20), encephalomyocarditis virus (21), and picornavirus (22), utilize components of the autophagy pathway to promote viral replication. Some of the viruses use the autophagosome as a membrane structure to support its replication as well as to hijack it as a mechanism for releasing the packaged virions in a non-lytic manner. This promotes the fusion of the autophagosome with the plasma membrane (12,19,21,23,24). While viruses promote autophagy induction, it has been shown that they have developed mechanisms to inhibit the maturation of autophagosomes by preventing the fusion between the autophagosome and the lysosome (19,20). However, recent findings also indicate that some RNA viruses benefit from autophagosome maturation. For example, acidification of the autophagosome is required for poliovirus particle assembly and virion maturation (18).

Our study showed that autophagy could be induced in HepG2 cells when the HBV replication became persistent in the cells, and this autophagy could be further enhanced by nutrient starvation. This is consistent with the previous findings that HBV induces autophagy in host cells *via* its HBx protein (14). Several studies showed that HBx interacts with C3-PI3K to initiate the autophagy process and the induction of autophagy is required for HBV DNA replication *in vitro* (14). *In vivo*, blocking autophagy by knocking out Atg5 greatly reduced HBV DNA replication (25). Our data also suggested that the induction of autophagy was necessary for HBV gene expression. The expressions of HBcAg and Hepatitis DNA polymerase were reduced when the autophagy was blocked by 3-MA, although they were not further increased when the enhanced autophagy was induced by starvation in addition to HBV. However, the expression of HBsAg was a little different. We found that inhibition of autophagy by 3-MA had little effect on the expression of HBsAg, whereas starvation led to the increase of HBsAg expression. It is not clear why the modulation of HBsAg expression is different. As

it is known that HBsAg expressions in active hepatitis patients were found in a few discrete hepatocytes with a diffuse cytoplasmic distribution, it is possible that HBsAg expression is not directly related to viral replication cycle (26).

Unlike several RNA viruses like HCV, HBV does not appear to replicate in autophagosome. We found that viral proteins, such as HBcAg, hepatitis B polymerase, or HBsAg did not localize in the autophagosome; *i.e.* there was no co-localization between viral proteins and autophagosome marker LC3. This observation suggests that HBV may not replicate and assemble in the autophagosome.

In contrast to previous observations, we found that SQSTM1/p62 level was reduced in HepG2.2.15 cells, and was further reduced in cells cultured in starvation condition (14,27). Our data indicated that the fusion of autophagosome with lysosome was not blocked in HBV infected cells. Part of the reason for this discrepancy may possibly due to the use of different hepatoma cell lines in our study. It is also possible, particularly in this case that HBV does not need use autophagosome as replication location, that there might be certain mechanisms that virus can selectively block protein degradation in the autophagosome based on its own needs.

This study reconfirmed several previous findings but the exact role of autophagy in HBV life cycle is still not clear. Sir *et al.* reported that autophagy could be induced by HBx protein and was required for efficient viral DNA replication; however, blocking the fusion of autophagosome and lysosome lead to reduced level of viral RNA but had little effect on viral DNA production (14). Li *et al.* showed that autophagy was needed for enveloped virion production at the ER, but had only slight effect on HBV DNA replication (27). Lazar C *et al.* reported that ER degradation-enhancing, mannosidase-like proteins (EDEM) were up-regulated in HepG2.2.15 cells, and the presence of viral surface proteins and EDEM1 led to the degradation of envelope proteins through autophagy. They suggested that it might be the mechanism for HBV to control the level of virions produced in infected cells and to establish chronic infection (28).

In summary, our study suggested that autophagy was induced by HBV, and was required for the viral proteins HBcAg and hepatitis B polymerase expression. However, HBV did not block the protein degradation through an autophagy pathway, and neither did it use the autophagosome as its site for DNA replication or virion assembly. Whether the protein degradation through autophagy pathway plays any role in facilitating the HBV life cycle needs further investigation.

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