Hepatitis B virus dampens autophagy maturation via negative regulation of Rab7 expression

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Summary
Hepatitis B virus (HBV) infection brings a huge challenge for medical health practitioners. It has been reported that invaded HBV escapes autophagic degradation through inhibiting lysosome maturation following enhanced autophagy formation, which putatively contributes to HBV replication and infection. However, the underlying mechanism by which HBV escapes from autophagic degradation remains elusive. In this study, we monitored the autophagic process using HepG2 cells and mice without or with transient HBV DNA plasmid transfection (pHepG2) or stable HBV infection (HepG2.2.15 cells) in vitro and in vivo. The results of Western blot, transmission electron microscopy and confocal microscopy, confirmed that HBV induced autophagy, while the fusion of autophagosomes with lysosomes was arrested. Furthermore, Rab7, a small GTPase that functions as a molecular switch responsible for the autophagosome-lysosome fusion, was inhibited, suggesting a potential mechanism for HBV-induced inhibition of autophagic degradation. In conclusion, our study proposes a potential mechanism for how HBV escapes autophagic degradation, which might be a novel therapeutic target for controlling HBV infection.

Keywords: HBV, Rab7, Autophagy, Autophagosome-lysosome fusion

1. Introduction
Hepatitis B virus (HBV) infection threatens about 248 million individuals' health globally. It remains a dreadful public health problem worldwide although the pervasive application of effective vaccination limits the number of newly HBV-infected patients dramatically (1). The availability of potent antiviral drugs decreases the morbidity and mortality inflicted by HBV infection. Nevertheless, almost all the current therapeutic measures are unable to completely eradicate the HBV DNA even after long-term antiviral therapy (2), and HBV could be reactivated when host immunity is compromised. The persistence of HBV DNA in the nucleus of infected hepatocytes is one of the intractable enigmas in approaches for the cure of chronic hepatitis B (CHB) (3). A better understanding of this refractory challenge may help to develop reliable priorities against HBV infection.

Macroautophagy (hereafter autophagy), a highly conserved cellular self-digestive process, plays a pivotal role in the degradation of dysfunctional proteins and...
damaged organelles to maintains cellular homeostasis or responses to harmful stimuli in higher eukaryotes (4). Autophagy initiates with the formation of an autophagosome, following docking and fusion with a lysosome. Only after the fusion can the contents of the autophagosome be degraded (5). Moreover, accumulating evidence shows that autophagy is also involved in many pathophysiologic processes of human diseases including microbial infection (6). For HBV, Sir D et al. first reported that HBV could activate the early autophagic pathway (7). But it seems like autophagy causes no harm to HBV (8). The precise underlying mechanisms why the enhanced autophagosomes promote HBV DNA replication instead of engulfing it need to be elucidated.

Rab7, a small GTPase of Rab superfamily, functions as a key organizer of multiple membrane trafficking processes. It has been well established that Rab7 serves as a master regulatory component for the biogenesis of autophagosomes, lysosomes and other lysosome-related organelles (9). More recently, Rab7 has been implicated in the maturation of autophagosomes and their fusion with lysosomes. Hampered fusion of autophagosomes and lysosomes caused by deficiency of Rab7 has been covered in mycobacterium tuberculosis and human parainfluenza virus infection (10, 11). Nevertheless, it is still uncertain whether Rab7 also plays an indispensable role in HBV-induced incomplete autophagy.

In the study, HBV infection induced autophagy was investigated, and the potential molecular mechanism that hindered autophagosome-lysosome fusion in HBV induced incomplete autophagic degradation was further explored.

2. Materials and Methods

2.1. Cell culture

HepG2 cells were obtained from the American Type Culture Collection (ATCC) and maintained according to ATCC instructions. HepG2.2.15 cells, which contained a stably integrated 1.3-copy HBV genome and could support persistent replication of HBV and produce intact HBV particles (12), was provided by the Department of Infectious Diseases of Changzheng Hospital. It was maintained in Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Invitrogen), 380 µg/mL G418 (Sigma), 125 µg/mL penicillin and 50 µg/mL streptomycin at 37°C in a humidified incubator containing 50 mL/L CO₂ (13).

2.2. Plasmids and cell transfection

HBV DNA plasmid (pHBV), which contains the 1.3-mer overlength HBV genome, and backbone plasmid pUC19 were a kind gift of professor James Ou from University of Southern California, Los Angeles, CA (14). Transient transfection into HepG2 of pHBV (pHepG2) or the backbone plasmid pUC19 (as the mock-transfected HepG2) were performed using FuGene 6 reagent (Roche) according to the manufacturer's instructions, respectively. HepG2 cells were lysed for both protein and RNA analysis after 48 h transfection. Meanwhile, level of HBsAg in the cell supernatant was determined using the ARCHITECT i2000SR HBsAg QT assay (Abbott). The cut-off value for determining if HBsAg is positive is S/N ratio ≥ 0.05, and the reporting units are S/N ratio. Level of HBV DNA in the cell supernatant was detected using the Roche COBAS HBO Amplicor MonitorTM assay. Tandem mRFP-GFP-LC3 expressing plasmid ptfLC3 (Addgene) was obtained from Addgene (15), and transfection was performed using Lipofectamine 2000 (Invitrogen).

2.3. Animal analysis

C57BL/6 mice (8 weeks old, male) were injected via the tail vein with phosphate buffer saline (PBS) in a volume that was equivalent to 8% of the body weight. The PBS with or without 10 µg of pHBV was delivered within 5 to 8 sec. Four days later, mice were sacrificed and liver tissues or serums were collected. Total liver protein was analyzed. Serum levels of HBsAg and HBV DNA of the mice were determined. The animal experiments were approved by the Ethics Committee of Ruijin Hospital.

2.4. Immunoblotting

Cells were directly lysed with RIPA containing protease and phosphatase inhibitors (Roche) and proteins were separated by SDS-PAGE after denaturation (16). Immunoblot analysis was performed by initial transfer of proteins onto polyvinylidenefluoride filters using a Mini Trans-Blot (Bio-Rad) followed by a blocking step with 5% nonfat dried milk plus 0.1% Tween 20 for 2 hours at room temperature and exposed to primary antibodies diluted 2,000-fold that recognized microtubule-associated protein light chain 3 (LC3, Cell Signal), autophagy-related protein 5 (Atg5, Cell Signal), Rab7 (Cell Signal), or 5000-fold for GAPDH (Santa Cruz) overnight at 4°C and subsequently washed. The blots were then incubated with a secondary antibody conjugated with Horse Radish Peroxidase diluted 5000-fold for 1 hour at room temperature. Signals were detected by a FluorChem E system (Alpha Innotech Corp). For some immunoblots, band densitometry was quantified using ImageJ software (National Institutes of Health).

2.5. RT-PCR

RT-PCR for Rab7 was performed as previously described (17), the primer pairs used in the study were as follows: Forward: 5'-ATGACCTCTAGGAAGAAAGTGTTGC TG-3', Reverse: 5'-TCAGCAACTGCAGCTTTCTGCC
HBV DNA stimulates the formation of autophagosomes

3. Results

3.1. HBV DNA stimulates the formation of autophagosomes

In order to study whether HBV DNA induces autophagy, we first established an HBV infection cellular system. In our system, the levels of HBsAg and HBV DNA in cell supernatant were monitored. Compared to the HepG2 control and the mock-transfected HepG2, HepG2.2.15 and pHBV showed significantly higher levels of both HBsAg and HBV DNA (Figure 1A, B). Atg5 and LC3, two pivotal ubiquitin-like proteins that are involved in the initiation of autophagy, serve as hallmarks of the formation of autophagosomes.

3.2. HBV infection induces autophagosome accumulation in vivo

To decipher whether HBV infection could induce autophagosome formation in vivo, pHBV was injected into C57BL/6 mice to simulate infection of HBV. Compared with those in the control, serum HBsAg and HBV DNA levels significantly increased in the pHBV injection group (Figure 2A and B). Histology showed disorganized hepatic lobules and lymphocytic infiltration in portal tracts, which indicated obvious inflammation in liver tissue (Figure 2C). Higher expression of LC3-II and Atg5 protein levels were observed in liver tissue of pHBV transfected mice than that of control mice (Figure 2D). Thus, our data indicates that HBV infection induces the formation of autophagosomes in vivo.

3.3. Autophagy maturation is hampered in pHepG2 cells

Autophagy maturation (docking and fusion of lysosome) is a key step for microbial degradation and is susceptible to pathogen hijacking. To uncover the underlying mechanism by which HBV induced autophagy promoted HBV replication instead of engulfing it, HepG2 cells were transfected with pHBV, followed by transfection with mRFP-GFP-LC3 plasmid. GFP is susceptible to

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Figure 1. HBV DNA induces autophagosome accumulation. HepG2 cells were starved, transfected with pHBV or pUC19 for 48h, respectively. (A, B) Levels of HBsAg and HBV DNA in supernatant of HepG2, mock-transfected HepG2, pHepG2 and HepG2.2.15 were measured. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. (C) The Atg5 and LC3 levels in HepG2, starvation treated HepG2, mock-transfected HepG2, pHepG2 and HepG2.2.15 were assessed by Western blot. (D) The bands density ratios of Atg5/GAPDH or LC3-II/GAPDH were determined by Western blot, and the results of control HepG2 was taken to be 100%. It was calculated as the average value of the data obtained from 3 independent experiments. *, $p < 0.05$. (E) Autophagic vacuoles in HepG2 and pHepG2 were observed by electron microscopy. The arrow indicates autophagosome, “m” represents mitochondria, and “n” represents nuclear.

Figure 2. pHBV increases autophagosome formation in mice. Male C57BL/6 mice (8 weeks old) were randomly injected with (pHBV, $n = 3$) or without (control, $n = 3$) 10 μg of pHBV. Ninety-six hours later, mice were sacrificed and liver tissues or sera were collected. (A and B) Mice serum HBsAg and HBV DNA levels were measured. **, $p < 0.01$; ***, $p < 0.001$. (C) Liver tissues of mice in control and pHBV-treated mice were stained with hematoxylin-eosin staining. (D) Atg5 and LC3 levels in mice liver tissue were assessed by Western blot.
acidified conditions, while mRFP signals are relatively stable, so the autophagosome is displayed as yellow punctate dots (both red and green) before fusion with lysosome, and red for mature autolysosomes. As shown in Figure 3A, yellow punctate dots were more obvious in pHepG2 cells than HepG2, while only sporadic red punctate dots were observed, confirming dominant presence of autophagosomes (yellow punctate dots), with rather few autolysosomes (red punctate dots). Cell numbers of HepG2 with autophagy were significantly increased after transfection with pHBV (Figure 3B). Above all, our data indicates inhibitory effects on autophagy maturation.

3.4. HBV infection induces reduction of Rab7

Small GTPase Rab7 has been considered as a master molecular switch during the formation of autolysosomes (20). Consequently, the expression of Rab7 was investigated to determine the role of Rab7 in HBV induced autophagy. Our results showed significant drops in Rab7 protein expression in pHepG2 cells and HepG2.2.15 cells (Figure 4A, B), as well as in Rab7 mRNA level (Figure 4C, D). Thus, it shows that HBV infection inhibits Rab7 expression and dampens autophagy maturation.

4. Discussion

Autophagy, a conserved cellular protective mechanism, has been regarded as a key component of the innate immune system (21). Induction of autophagy results in the elimination of invading pathogens, whereas some microbes have evolved strategies to manipulate autophagy for their own benefit. Several reports have proclaimed that HBV infection is able to induce autophagy (7, 22, 23), and transfection of pHBV in hepatic and hepatoma cell lines both promote formation of autophagosomes (23). Consistently, our results also discovered increased autophagy in pHepG2 cells and HBV-expressing HepG2.2.15 cells. Furthermore, the in vivo results support our conclusion when transfecting C57BL/6 mice with pHBV.

HBV-induced autophagy have been extensively investigated, and the views of its effect on HBV and chronic infection were still elucidated. Several studies suggested that HBV could manipulate autophagy to affect virus production and chronic infection.
establishment (24, 25). On the contrary, Tian et al. indicated that incomplete autophagy could regulate HBV DNA replication instead of HBV virions in vivo (26). However, how autophagy might implement regulation and help pathogen escape from the following autophagic degradation still remained elusive. A recent study demonstrated that hepatitis B virus x protein impaired lysosomal degradative capacity by disturbing its acidification without influencing the fusion of autophagosomes and lysosomes (27). Intriguingly, one of the most important findings from our current study turned out that the fusion of autophagosomes and lysosomes was hampered in pHepG2 cells. This discrepancy might be due to transfection of a different pHBV in our experiment system. Besides, the effects of different cell lines also should be considered. Some intracellular pathogens, mycobacterium tuberculosis and human parainfluenza virus (10, 11), hijack autophagy and inhibit its maturation for their replication, prompting us to speculate that HBV might induce autophagosome formation as its replication site to facilitate the viral life cycle but block the following autophagosomes-lysosomes fusion to evade autophagic degradation.

Fusion proteins, such as VAMP1, 2, 7 and 8, Vti1b, syntaxin1, and 8, VPS28, LAMP2 and Rab7, play a key role in the fusion of autophagosomes and lysosomes. Our data suggested that a difference merely existed in Rab7 mRNA levels between HepG2 and pHepG2. Rab7, a late endosome-/lysosome-related small GTPase, may be the only lysosomal Rab protein identified so far (9), which functions as a crucial molecular switch in autophagy. Rab7 can act as a master factor to regulate the biogenesis and maintenance of lysosomes (28). Furthermore, blocking Rab7-mediated trafficking machineries downregulate autophagy-mediated clearance (29). Extensive research delineated that Rab7 could promote maturation of autophagosomes and regulate their fusion with lysosomes (30). However, abnormal expression of Rab7 has been implicated in invasion of many other pathogens. For example, Hu D et al. reported that Rab7 could be blocked to prevent autophagosome-lysosome fusion in mycobacterium tuberculosis infection (10). Hampered fusion of autophagosomes and lysosomes caused by deficiency of Rab7 has also been covered in human parainfluenza virus infection (11). Moreover, Inoue J et al. has reported that HBV could hijack Rab7 for its replication, as siRNA-mediated depletion of Rab7 dramatically augmented the secretion of virions (31). Accordingly, our data showed that HBV infection inhibited Rab7 expression in pHepG2 cells and HepG2.15 cells. Whereas, starvation treatment, which induced autophagy in the whole process, increased expression of Rab7 in HepG2 cells. Combined with these findings, our study made it clear that Rab7 might be responsible for the HBV-induced incomplete autophagic progress, which could convincingly show how HBV could enhance the formation of autophagosomes and escape subsequent autophagic degradation.

In conclusion, this study confirms that HBV induces autophagosome formation but blocks autophagy maturation. Moreover, it supports the idea that HBV dampens Rab7 expression, which is responsible for the autophagosome-lysosome fusion. Our findings help to clarify the role of Rab7 in HBV-induced incomplete autophagic degradation and provide a potential target for further manipulation of autophagy in the treatment of HBV infection.

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References


