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Autophagy induced by infectious hematopoietic necrosis virus inhibits intracellular viral replication and extracellular viral yields in epithelioma *papulosum cyprini* cell line

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ABSTRACT

Infectious hematopoietic necrosis virus (IHNV) is a common pathogen that causes severe disease in the salmonid aquaculture industry. Recent work demonstrated that autophagy plays an important role in pathogen invasion by activating innate and adaptive immunity. This study investigated the relationship between IHNV and autophagy in epithelioma *papulosum cyprini* cells. The electron microscopy results show that IHNV infection can induce typical autophagosomes which are representative structures of autophagy activation. The punctate accumulation of green fluorescence-tagged microtubule-associate protein 1 light chain 3 (LC3) and the protein conversion from LC3-I to LC3-II were respectively confirmed by confocal fluorescence microscopy and western blotting. Furthermore, the effects of autophagy on IHNV replication were also clarified by altering the autophagy pathway. The results showed that rapamycin induced autophagy can inhibit both intracellular viral replication and extracellular viral yields, while autophagy inhibitor produced the opposite results. These findings demonstrated that autophagy plays an antiviral role during IHNV infection.

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1. Introduction

Infectious hematopoietic necrosis virus (IHNV) is a rhabdovirus belonging to the genus *Novirhabdovirus* in the family *Rhabdoviridae* (Dixon et al., 2016). Like other rhabdoviruses, IHNV is a single-stranded, nonsegmented, negative-sense RNA virus that encodes five structural proteins: the RNA-dependent RNA polymerase L, glycoprotein G, matrix protein M, phosphoprotein P, nucleoprotein N, and a nonstructural protein nonvirion NV (Ammayappan et al., 2010; Ammayappan and Vakharia, 2011). IHNV is an important viral disease in salmonid aquaculture and outbreaks of IHN usually cause 80%–100% mortality (Ahmadivand et al., 2017; Breyta et al., 2013; Enzmann et al., 2005). Most research on IHNV has centered on studying vaccines, such as DNA vaccines (LaPatra et al., 2001; Xu et al., 2017), attenuated vaccines (Fryer et al., 1976; Ristow et al., 2000), inactivated virus vaccines (Anderson et al., 2008) and oral vaccines (Ballesteros et al., 2015; Zhao et al., 2017), while only a few studies have been conducted on the relationship between IHNV and host cells (Cao et al., 2016; Nita-Lazar et al., 2016; Wargo et al., 2017). Because understanding the pathogenesis of IHNV will impact the design and selection of methods to prevent infection with this virus, the study of host-virus interactions may lead to new strategies of protecting from IHNV infection.

Autophagy is a basic, highly conserved, homeostatic mechanism that delivers cytoplasmic proteins and damaged organelles as well as aggregated and long-lived proteins to lysosomes for degradation and recycling by a membrane-mediated process (Mizushima and Komatsu, 2011; Ouimet, 2013; Reggiori, 2006). Many previous studies have revealed that autophagy also acts as a defense mechanism in various pathogen (bacteria and viruses) infections and plays a role in the clearance of these pathogens (Li et al., 2016; Moreau et al., 2015; Moy et al., 2014). The most likely mechanisms by which autophagy inhibits pathogen replication and secretion may be degrading pathogen proteins by activating the autophagy

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pathway or through innate and adaptive immune responses (Levine et al., 2011; Richetta and Faure, 2013; Schmid and Munz, 2007). However, some pathogens induce autophagy activation to participate in their own replication, such as classical swine fever virus (Pei et al., 2013), hepatitis C virus, and dengue virus (Dreux et al., 2009). Now, there are some works on fish viruses that have been reported to induce autophagy, such as spring viraemia of carp virus (SVCV) (Liu et al., 2015), vesicular stomatitis virus (VSV), viral hemorrhagic septicemia virus (VHSV) (Garcia-Valtanen et al., 2014), infectious salmon anemia virus (ISAV) (Schiotz et al., 2010), infectious spleen and kidney necrosis virus (ISKNV) (Li et al., 2017), and snakehead fish vesiculovirus (SHVV) (Wang et al., 2016). But the relationship between autophagy and different fish virus are distinguished. Clarifying the relationship between autophagy and pathogens may reveal new strategies for reducing bacteria and virus infection by modulating autophagy.

Around 36 autophagy genes, called *ATG*, have been identified as participating in different autophagy stages (Klionsky, 2007; Reggiori, 2006). Microtubule-associate protein 1 light chain 3 (LC3) participates in cargo recruitment and autophagosomes biogenesis, and LC3B has been widely used as a marker for autophagic structures (Hosseini et al., 2014). After synthesis, nascent LC3 is cleaved by Atg4 and then covalently bonds with phosphatidylethanolamine (PE) at the C-terminal fragment to become LC3-II on the autophagosomal membrane. LC3-I is located in the cytoplasm, while LC3-II is located at the inner and outer membranes of autophagosomes. The conversion from LC3-I to LC3-II typically serves as an identifying characteristic for autophagy activity (Mizushima et al., 2010).

To our knowledge, there are no previous reports on the relationship between IHNV and autophagy. Given its importance as highlighted above, we investigated this relationship in the present study. By detecting the formation of autophagic vesicles and the key autophagy molecules, we assessed if IHNV infection could induce autophagy in using epithelioma *papulosum cyprini* (EPC) cells. Furthermore, we also clarified the relationship between autophagy and IHNV replication by altering the autophagy pathway with an autophagy inhibitor or enhancer. The resulting findings on the relationship between IHNV and autophagy may provide insight for new anti-IHNV strategies that modulate autophagy.

2. Materials and methods

2.1. Cell culture and virus infection

EPC cells (CRL-2872, ATCC) were kindly provided by Dr. Zeng Lingbing, Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Wuhan, China. The IHNV virus strain was isolated in our laboratory from infected rainbow trout. EPC cells were cultured at 25 °C with Eagle's minimum essential medium (MEM, Gibco, Grand Island, New York, USA) containing 0.1 mg/ml penicillin and streptomycin with 10% fetal bovine serum (FBS, Gibco). The monolayers of EPC cells were infected with IHNV at a multiplicity of infection (MOI) of 0.1, and the infected cells were then cultured in MEM medium with 2% FBS for viral propagation at 15 °C.

2.2. Reagents and antibodies

Anti-p62/SQSTM1 antibody (P0068), anti-LC3B antibody (L7543), rapamycin (R8781), and 3-methyladenine (3-MA) (M9281) were purchased from Sigma (St. Louis, Missouri, USA). Anti-β tubulin antibody (ab 179513), Cy3-tagged anti-rabbit IgG antibody (ab97075), and HRP-conjugated goat anti-rabbit IgG (ab6721) were purchased from Abcam (Cambridge, United Kingdom). The rabbit polyclonal antibody used against IHNV glycoprotein (G) was researched and developed by our laboratory.

2.3. Construction of the pEGFP-LC3 plasmid

TRIzol Reagent (15596026, Invitrogen, Carlsbad, California, USA) was used under the manufacturer's instructions for isolating the total RNA from EPC cells. The *LC3* gene sequence was amplified with a PrimeScript One Step RT–PCR kit (RR057A, Takara, Japan), according to the manufacturer's instructions. After being sequenced, the *LC3* gene was ligated to the pcDNA3.1 plasmid, which already has an *EGFP* gene, to construct the pEGFP-LC3 plasmid.

2.4. Transmission electron microscopy

Confluent monolayers of EPC cells were treated with either IHNV infection (MOI = 0.1), mock infection with PBS (negative control), or rapamycin (positive control). After incubation for 24 h, the treated cells were washed three times with phosphate-buffered saline (PBS) and collected. Cells were fixed with PBS containing 2.5% glutaraldehyde for 24 h at 4 °C, after which they were post-fixed in PBS containing 1% osmium tetroxide for 70 min at 25 °C and then dehydrated in ethanol. Following dehydration, the cells were rinsed with acetone and embedded in resin for later sectioning. The samples were stained with uranyl acetate-lead citrate and observed using a Hitachi 7650 (Japan) transmission electron microscope.

2.5. Confocal fluorescence microscopy

Confluent monolayers of EPC cells were transfected with 2 μg of pEGFP-LC3 plasmid using Lipofectamine 2000 Reagent (1857332, Invitrogen). At 24 h post-transfection, the cells were treated with either IHNV (MOI = 0.1), PBS (negative control), or rapamycin (500 nM; positive control) and then fixed with 4% paraformaldehyde for 30 min. Following fixation, the cells were permeabilized with 0.5% Triton X-100 for 20 min. After being washed three times with PBS, the cells were successively incubated with anti-G antibody for 1 h and Cy3-tagged anti-rabbit IgG antibody for another 1 h. Nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI) for 5 min. A DeltaVision microscope imaging system (GE Healthcare, USA) was used for image observation.

2.6. Western blotting

Cells were washed three times with PBS and lysed in RIPA (P0013B, Beyotime, China) with 1 mM PMSF (ST505, Beyotime). Cellular lysates were separated by 12% SDS-PAGE and then transferred onto a nitrocellulose membrane (BS-NC-45, Biosharp, China) followed by blocking with 5% skim milk in PBST (PBS with 0.05% Tween) for 1 h at 37 °C. The membrane was incubated with primary antibody, either anti-LC3B (1:1000 dilution), anti-G (1:1000 dilution), anti-p62 (1:1000 dilution), or anti-β tubulin (1:1000 dilution), for 12 h at 4 °C. After being washed three times with PBST, the membrane was incubated with an HRP-tagged antibody (1:3000 dilution) for 1 h at 25 °C. The images were observed with enhanced chemiluminescence (ECL) solution (34077, Thermo Scientific, Rockford, Illinois, USA) using a ChemiScope 6000 Touch (Clinx, Shanghai, China), and quantification of the protein bands was performed using Clinx Chemi Analysis software.

2.7. Cell viability assay

The cell viability assay was performed and assessed using a previously described method (Zhang et al., 2011). Briefly, EPC cells

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