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Autophagy inhibits viral genome replication and gene expression stages in West Nile virus infection

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ABSTRACT

Autophagy is a lysosomal degradation pathway that is implicated in many viral infections. However, its role in West Nile virus (WNV) infection remains controversial. In the present study, we examined the relationship between WNV infection and autophagy in infected cells. We demonstrated that LC3-II expression, a molecular marker for autophagosomal membranes, was enhanced in WNV-infected cells 6 h post-infection. LC3-II expression was further enhanced in WNV-inoculated cells when treated with a lysosomal protease inhibitor. Meanwhile, WNV replication in cells lacking *Atg5*, an essential factor for autophagy, was increased compared with replication in wild-type cells. In addition, WNV replication was inhibited in cells lacking *Atg5* when they were transfected with an ATG5 expression plasmid. These results suggest an antiviral role for autophagy in WNV-infected cells. We also examined which viral replication stages were affected by autophagy by using a Tat-beclin 1 peptide to induce autophagy and pseudo-infectious WNV reporter virus particles (WNV-RVPs) that monitor viral genome replication and gene expression stages *via* GFP expression. We found that autophagy induction in HeLa cells by Tat-beclin 1 peptide 3 h after WNV inoculation inhibited viral replication, and GFP expression was significantly inhibited in wild-type cells when compared with cells lacking *Atg5*. Taken together, these results suggest that autophagy is induced by WNV infection, and that this induction inhibits WNV replication at the viral genome replication and gene expression stages.

1. Introduction

West Nile virus (WNV) is a single-stranded, positive-sense RNA virus in the *Flaviviridae* family that causes West Nile encephalitis and death in humans and horses (Kobayashi et al., 2012; Suthar et al., 2013). The viral genome encodes a single polyprotein that is processed by host and viral proteases into three structural proteins (C, prM, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Samuel and Diamond, 2006). To initiate the WNV replication life cycle, WNV attaches to host cell receptor(s). Thereafter, WNV is endocytosed, fused with the endosomal membrane, and the viral genome is delivered into the cytoplasm (Makino et al., 2014; Medigeshi et al., 2008). Viral RNA and protein synthesis occurs in association with endoplasmic reticulum-associated membranes (Fernandez-Garcia et al., 2009). In flavivirus infection, these stages take place as early as 3 h post-infection (hpi) (Lindnbach et al., 2007). Synthesized structural proteins and viral RNA are assembled and transported through the host secretory pathway, and infectious virions are released from the infected cell by exocytosis (Samuel and Diamond, 2006). WNV replication appears dependent on the availability of host factors to interact with viral proteins, but the interaction between host factors and viral proteins is not fully understood.

Macroautophagy (herein referred to as autophagy) is a highly conserved, intracellular degradation system (Dong and Levine, 2013). Cytoplasmic components are sequestered by autophagosomes and degraded upon fusion with lysosomes to maintain cellular homeostasis (Morishita et al., 2013). Genetic studies in

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yeast have identified a set of autophagy-related genes (*Atg*) genes that are required for autophagy (Mizushima et al., 2011). For instance, *Atg5* and *Atg12* are required for autophagosome formation (Mizushima et al., 1998, 2001). The presence of ATG5 and its proper conjugation with ATG12 are essential for elongation of the autophagic isolation membrane (Mizushima et al., 1998, 2001). In mammalian cells, autophagy is involved in starvation, quality control of intracellular proteins and organelles, and elimination of intracellular microbes including viruses (Dong and Levine, 2013; Shoji-Kawata and Levine, 2009).

The autophagic pathway can play an antiviral role by restricting the replication of some viruses, such as vesicular stomatitis virus and Chikungunya virus (Joubert et al., 2012; Shelly et al., 2009). Autophagic antiviral function may be summarized as the following: (1) digestion of intracytoplasmic viral components (virophagy); and (2) activation of innate and adaptive immunity by presentation of viral molecules (Dong and Levine, 2013). In contrast, some viruses such as poliovirus, dengue virus, and hepatitis C virus, have evolved mechanisms to escape host autophagy and use components of the autophagic machinery for replication (Dreux et al., 2009; Heaton and Randall, 2010; Jackson et al., 2005).

The role of autophagy in WNV infection is less clear, as conflicting results have been reported (Beatman et al., 2012; Shoji-Kawata et al., 2013; Vandergaast and Fredericksen, 2012). No difference in WNV replication in mouse embryonic fibroblasts (MEFs) lacking *Atg5* has been observed at a multiplicity of infection (MOI) of 3 or 0.1 (Beatman et al., 2012; Vandergaast and Fredericksen, 2012). However, the autophagy-inducing peptide, Tat-beclin 1, decreases WNV replication and reduces mortality in mice infected with WNV (Shoji-Kawata et al., 2013).

In the present study, we examined LC3-II expression, a marker for autophagosomal membranes, in WNV-infected cells. The effects of autophagy on WNV replication in *Atg5*$^{-/-}$ MEFs were evaluated to better understand the role of autophagy in WNV infection. Furthermore, we examined the WNV viral replication stages that are affected by autophagy using Tat-beclin1 and pseudo-infectious WNV reporter virus particles (WNV-RVPs) able to infect susceptible cells without producing progeny virions (Pierson et al., 2006), to address how autophagy is involved in WNV replication cycle.

2. Materials and methods

2.1. Cells and viruses

Human neuroblastoma cells (SK-N-SH cells) and Vero E6 cells were grown in minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 2 mM L-glutamine (Sigma, St. Louis, MO). HEK-293T cells were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% FBS. *Atg5*$^{+/+}$ and *Atg5*$^{-/-}$ MEFs (Kuma et al., 2004) were provided by the RIKEN BRC (Tsukuba, Japan) through the National Bio-Resource Project of the MEXT, Japan. HeLa cells were obtained from the Health Science Research Resource Bank (Osaka, Japan). *Atg5*$^{+/+}$ MEFs, *Atg5*$^{-/-}$ MEFs, and HeLa cells were grown in DMEM supplemented with 10% FBS, penicillin, streptomycin, and 2 mM L-glutamine. The WNV 6-LP strain, previously established by plaque purification of the WNV NY99-6922 strain isolated from mosquitoes in 1999 (Shirato et al., 2004a,b), was provided by Dr. I. Takashima (Hokkaido University, Japan). Indicated cell line cultures were infected with WNV at specified MOI. After 1 h at 37 °C, the inoculum was replaced with culture medium and cells were incubated at 37 °C. Cells and culture supernatants were harvested at indicated time points. All experiments with WNV were performed at the Biosafety Level-3 facility at Hokkaido University in accordance with institutional guidelines. *Atg5*$^{+/+}$ and *Atg5*$^{-/-}$ MEFs were infected with Herpes Simplex virus type-1 (F strain) at MOI = 0.01. After 1 h at 37 °C, the inoculum was replaced with culture medium and the cells were incubated at 37 °C. Cells and culture supernatants were harvested at the indicated time points.

2.2. Antibodies, plasmids, and reagents

Mouse anti-LC3, rabbit anti-LC3, and rabbit anti-ATG5 polyclonal antibodies were purchased from MBL (Nagoya, Japan). A rabbit anti-LC3 monoclonal antibody was purchased from Cell Signaling Technology (Beverly, MA). The anti-JEV serum that cross-reacted with the structural proteins of WNV was prepared from rabbits immunized by 2 intravenous inoculations of JEV (JaGAr-01 strain) (2×10^9 plaque-forming units/rabbit) (Kimura et al., 1994; Kobayashi et al., 2012). The mouse anti-actin monoclonal antibody was purchased from Millipore (Billerica, MA). For the expression of murine ATG5 in *Atg5*$^{-/-}$ MEFs, pCI-neo-mApg5 (plasmid 22956), and pCI-neo-mApg5 (K130R) (plasmid 22957) were obtained from Addgene (Cambridge, MA) (Mizushima et al., 2001). To make WNV-RVP, WNV structural genes were cloned into pCXSN, which was generated from pCMV-myc (Clontech, Mountain View, CA) by replacing the sequence of the myc tag and multicloning site with restriction enzyme sites *Xho*I, *Sal*I, and *Not*I (Kobayashi et al., 2013). The resultant plasmid was designated pCXSN-CME (Hasebe et al., 2010). The WNV replicon cDNA construct, pWNIIrep-GFP, was generously provided by Dr. Robert W. Doms (Pierson et al., 2006). E64d and pepstatin A, the lysosomal protease inhibitor, were purchased from the Peptide Institute (Osaka, Japan). The Tat-beclin1 peptide (YGRKKRRQRRRGGTNVFNATFEIWHDGEFGT) and the Tat-scrambled peptide (YGRKKRRQRRRGGVGNDFFINHETTG-FATEW) were synthesized and purified to 95% by Sigma as described previously (Shoji-Kawata et al., 2013). Peptides were dissolved in phosphate buffered saline (PBS) (20 mM) and stored at −80 °C. For peptide treatment, cells were washed with PBS and treated with peptides (10 μM) dissolved in OPTI-MEM (Life Technologies, Rockville, MD) acidified with 0.15% (v/v) 6 N HCl.

2.3. Immunocytochemistry

To observe viral antigen positive cells, SK-N-SH cells were seeded at 200,000 cells per well in a 24-well dish, in which each well contained a 12-mm cover glass, and the cells were then WNV inoculated (MOI = 0.01) for 6 h. The WNV infected and mock-infected cells were fixed with 4% paraformaldehyde for 10 min, before washing with PBS. The cells were permeabilized in 100 μg/ml digitonin for 15 min, blocked with 1% bovine serum albumin (BSA)-PBS, and then stained with mouse anti-LC3 monoclonal antibody and anti-JEV rabbit serum in 1% BSA-PBS for 3 h at room temperature. The immune complexes were visualized by incubating with either Alexa Fluor 488-conjugated or Alexa Fluor 594-conjugated secondary antibodies (Life Technologies). The cells were then observed using a Zeiss 780 LSM confocal microscope (Jena, Germany). Immunopositive foci were confirmed by observing each cover glass. The number of punctate structures of LC3 per cell was counted from different microscopic fields.

2.4. Immunoblotting

For immunoblotting, cells were harvested at indicated time points after WNV infection and transfection, lysed in TNE buffer [10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100] supplemented with Complete Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN). Cell lysates were fractionated by SDS-PAGE, and separated proteins were