The *in vitro* effects of Herpes simplex virus and Rubella virus on autophagy

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INTRODUCTION

Viruses, during the course of their replication, alter several important intracellular processes. These changes may trigger various regulated cell death subroutines, such as apoptosis, anoikis, necroptosis, parthanatos, pyroptosis and autophagy. Cell destruction plays an important role in the pathogenesis of viral infections, and also affects the development of the clinical symptoms.

Autophagy and its role in cell homeostasis

In eukaryotic cells, three types of autophagy have been described: *(i)* microautophagy, *(ii)* chaperone-mediated autophagy, and *(iii)* macroautophagy.

In microautophagy, portions of the cytoplasm are engulfed by the lysosome directly.

In chaperone-mediated autophagy, unfolded proteins are translocated directly across the limiting membrane of the lysosome.

In macroautophagy (hereafter referred to as autophagy), portions of the cytoplasm and organelles are sequestered into double-membrane vesicles termed autophagosomes, and delivered to lysosomes by vesicular transport. Macroautophagy is induced by nutrient deprivation, cellular stress, infections or rapamycin treatment via complex signaling pathways. The major regulator of macroautophagy is the mTOR (mammalian target of rapamycin) protein. The mTOR senses the nutrient supply, energy status and growth factors, and inhibits the induction of autophagy. Environmental stressors, starvation or rapamycin treatment inhibit the mTOR, and thereby trigger an increase in autophagic activity. Following the induction of autophagy, inhibition of the mTOR leads to the activation of the ULK1 [autophagy-related gene 1 (Atg1) homolog] complex, which in turn undergoes translocation to a certain domain of the endoplasmic reticulum (ER). Once in the ER, the ULK1 complex activates the class III phosphatidylinositol 3-kinase (PI3K) complex. The activated PI3K phosphorylates the ER lipid phosphatidylinositol at position 3 of the inositol ring. Phosphatidylinositol 3-phosphate then recruits double FYVE-containing protein 1 (DFCP1) and promotes the formation of the omegasome from which the isolation membrane appears to be generated. The isolation membrane provides a platform for two ubiquitin-like conjugation systems implicated in the elongation step. The first conjugation system, consisting of Atg7 (E1-like enzyme) and Atg10 (E2-like enzyme), triggers the covalent conjugation of Atg12 to Atg5. The Atg12–Atg5 conjugate binds Atg16 and forms an E3-like enzyme. The second conjugation system, consisting of Atg7 (E1-like enzyme) and Atg3 (E2-like enzyme), triggers the covalent conjugation of phosphatidylethanolamine to the soluble microtubule-associated protein 1 light chain 3-I (LC3-I), leading to the formation of LC3-II. The Atg12–Atg5–Atg16 supercomplex is required for the incorporation of LC3-II to the isolation membrane. Accumulation of the lipidated form of LC3 is essential for the expansion and closure of the autophagosome membrane. Since LC3-II persists in the autophagosome membrane even after the autophagosome – lysosome fusion, it is an excellent marker for detecting autophagy. Maturation is the final, degradative stage of the process, when closed autophagosomes fuse with late lysosomal organelles, generating autolysosomes in which the engulfed material is then degraded. Selective autophagy is a special form of macroautophagy in which misfolded proteins labeled with ubiquitin or pathogenic microorganisms decorated with ‘eat me’ signals are captured selectively by cargo receptors, such as p62/sequestosome-1 (SQSTM1), neighbor of BRCA1 gene 1 protein (NBR1), nuclear domain 10 protein 52 (NDP52)

and optineurin. The adaptors bind to LC3 via a domain known as LIR (LC3 interacting region), and deliver their substrates to autophagosomes.

Autophagy plays important roles in numerous physiological processes and pathological conditions, including infections. Autophagic capture and delivery of viruses to lysosomes can severely compromise viral replication, and may function as an ancient antiviral defense mechanism known as xenophagy. Several viruses have evolved different strategies to elude the destructive power of autophagy. Some viruses can prevent autophagic recognition, block autophagosomal initiation and maturation or inhibit the fusion of lysosomes with autophagosomes, while others utilize autophagy to their own advantage.

Rubella virus and its interaction with the infected cells

Rubella virus (RV) is the sole member of the *Rubivirus* genus in the *Togaviridae* family. RV is an enveloped virus with a positive-sense, single-stranded RNA genome. RV virions are spherical particles with a diameter of 55 - 90 nm. The RV nucleocapsid has icosahedral symmetry. The genome contains two open reading frames (ORFs). The 5' ORF encodes the two non-structural proteins (p150 and p90). p150 possesses an ADP-ribose-binding domain, a sequence with a proline-rich region, and motifs for methyltransferase and papain-like cysteine protease, while p90 comprises domains for replicase and helicase. The 3' ORF encodes the structural proteins, including the capsid protein, and also the E1 and E2 envelope glycoproteins. The most characteristic feature of RV is that its intracellular life cycle is closely connected to the endomembrane system. While RV establishes persistent, noncytocidal infection in many cell types, it can also trigger apoptotic cell death in some cell lines. The ability of RV to induce apoptosis varies considerably with cell type and virus strain. Interesting results indicate that RV activates multiple pathways of apoptosis. However, the effect of this virus on other cell death subroutines has not yet been investigated.

RV can cause postnatal and congenital infections. Postnatal RV infection, known as rubella disease or German measles, is one of the five classic childhood diseases. Postnatal rubella is characterized by maculopapular rash, lymphadenopathy and fever. In seronegative or immunodeficient pregnant women, the virus can cross the placenta and infect the fetus during maternal viremia. RV is capable of spreading widely throughout the developing fetus, and almost any organ can be infected. A chronic and generally non-cytolytic infection is then established. By disturbing the ontogeny of fetal organs, congenital RV infection may lead to serious developmental abnormalities, collectively termed the congenital rubella syndrome (CRS). Common clinical manifestations of CRS include congenital heart defects (most frequently patent ductus arteriosus, pulmonary artery or valvular stenosis), cataract, glaucoma or retinopathy, hearing loss, psychomotor retardation, skin rash at birth, low birth weight and hepatosplenomegaly.

Herpes simplex viruses and their interactions with the infected cells

Herpes simplex viruses 1 and 2 (HSV-1 and -2) belong to the *Herpesviridae* family. HSV-1 and HSV-2 particles are 120-200 nm in diameter and have four structural elements: *(i)* the core, *(ii)* an icosahedral capsid containing 162 capsomeres, *(iii)* the tegument and *(iv)* an outer envelope with glycoprotein spikes. The core is composed of linear double-stranded DNA of the virus. The genomes of HSV-1 and HSV-2 contain two covalently-linked sections, termed long unique region (U_L) and short unique region (U_S), each of which is bracketed by two sets of inverted repeats of DNA. Following

entry, HSV DNA may become circularized by cellular DNA repair enzymes, and establish a latent infection within the neurons of the sensory ganglia, brain stem, olfactory bulbs and temporal lobe. During latency no viral particles are formed, only the latency-associated transcript is expressed, and the viral genome is maintained in an episomal state. From this cellular reservoir, reactivation may occur. In other cell types, viral DNA remains linear, and a productive replication cycle ensues. Initially, IE or α genes are transcribed, and their protein products transactivate the early or β and late or γ genes. The products of early genes are primarily implicated in the replication of HSV DNA. The late genes encode structural components of the HSV virion. Viral DNA replicates in the nucleus by a rolling circle mechanism, leading to the formation of concatemeric genomes, which in turn are cleaved and packaged into preformed capsids. Following virion maturation, tegumented capsids gain their envelope by budding through the Golgi apparatus, and then released from the cell by reverse endocytosis. HSV-1 and HSV-2 are capable of infecting different histological types of cells, including epithelial cells, fibroblasts, neurons of sensory ganglia and central nervous system, lymphocytes, monocytes, and dendritic cells. The fate of infected cell largely depends on its histological type. Neurons, in which HSV-1 and HSV-2 establish latency, survive. Epithelial cells, fibroblasts, monocytes and dendritic cells die primarily by way of necrosis and apoptosis. Moreover, both HSV-1 and HSV-2 have the capability to modulate the process of autophagy.

HSV-1 and HSV-2 invade the body through the skin and mucous membranes of the orofacial or genital regions.These viruses can cause a variety of mild and severe diseases, including herpes simplex labialis, ocular disease, genital herpes and encephalitis. HSV can also be contracted *in utero*, at the time of delivery or postnatally, leading to neonatal infection. Neonatal herpes is severe, and has a tendency for dissemination to the central nervous system and visceral organs.

AIMS

I. Investigation of the effect of RV on autophagy in the SIRC cell line
II. Investigation of the effects of HSV-1 and HSV-2 on autophagy in the SIRC cell line

MATERIALS AND METHODS

Cell cultures

SIRC cell line: The Statens Seruminstitut Rabbit Cornea (SIRC) cell line (obtained from ATCC, code CCL-60), was grown in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% fetal calf serum.

Vero cell line: The Vero cell line was grown in Dulbecco's modified Eagle's minimal essential medium supplemented with 7.5% fetal calf serum. Both cell lines used were cultured at 37 °C in a 5% CO_2 atmosphere.

Viruses

RV: The To336 strain of RV (obtained from ATCC, code VR-553) was propagated at an MOI of 0.01 PFU/cell in Vero cell cultures for 7 days at 35 °C. The culture fluid of RV-infected Vero cells was harvested, stored at –70 °C, and used as infecting stock of the virus.

HSV-1 and HSV-2: The KOS strain of HSV-1 and the wild-type HSV-2 were propagated at a multiplicity of infection (MOI) of 0.001 plaque forming unit per cell (PFU/cell) in Vero cell cultures for 3 days at 37 °C. The culture fluids of HSV-1- or

HSV-2-infected Vero cells were harvested, stored at –70 °C, and used as infecting stock of the virus.

Methods used to detect viral replication

RV: Virus plaque assay was performed on confluent monolayers of Vero cells inoculated with virus solution for 1 hour (h) at 35 °C and overlaid with 0.5% agarose in phenol red-free Eagle's minimum essential medium supplemented with 7.5% FCS and 2 mM L-glutamine. After 4 days of culturing at 35 °C, a second agarose overlay containing 0.005% neutral red was added. Plaque titers were determined at 7 days postinfection (dpi). For experiments, SIRC cell cultures were inoculated with the To336 strain of RV at an MOI of 5 PFU/cell.

HSV-1 and HSV-2: For experiments, SIRC cell cultures were inoculated with the KOS strain of HSV-1 or a wild-type strain of HSV-2 at different MOIs. Virus plaque assays were performed on confluent monolayers of Vero cells inoculated with one or other HSV solution for 1 h at 37 °C and overlaid with 0.5% agarose in phenol red-free Eagle's minimum essential medium supplemented with 7.5% FCS and 2 mM L-glutamine. After 2 days of culturing at 37 °C, a second agarose overlay containing 0.005% neutral red was added. Plaque titers were determined at 3 dpi.

Indirect immunofluorescence assay

Cytospin cell preparations were fixed in methanol-acetone (1:1) for 15 minutes (min) at 20 °C. The cells were treated with 2% bovine serum albumin in phosphate-buffered saline (PBS) for 30 min at 37 °C to block unspecific binding of the antibodies. To detect RV glycoprotein E1, HSV gD and LC3B, the slides were stained with primary antibodies for 1 h at 37 °C. After washing with PBS, the samples were reacted with fluorescein isothiocyanate (FITC)-conjugated or CF640R-labeled species-specific secondary antibodies for 1 h at 37°C. The cells were visualized by confocal microscopy using an Olympus FV 1000 confocal laser scanning microscope (Melville, NY, USA) or an epifluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany). The ratios of RV- and HSV-infected cells were determined after counting 1000 cells in random fields. LC3B-positive vacuoles were automatically quantified for each field after subtraction of the background level and establishment of an intensity threshold by using the Image J software. Both the numbers and the average surface areas of the LC3B-positive puncta were normalized to the numbers of cells in each field. An average of 780 cells were analyzed for each condition. The fluorescence intensity of LC3B was determined by using the line scan analysis function of the Image J software.

Acridin orange (AO) staining

Cytoplasmic acidification was assessed by the AO staining procedure of the autophagic vacuoles. The cells, grown on glass coverslips were incubated for 15 min at 37°C in serum-free medium containing 2 μg/ml 3,6-bis(dimethylamine)acridine. After washing with PBS, fluorescent micrographs were obtained by using an Olympus FV 1000 confocal laser scanning microscope (Melville, NY, USA) or an epifluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany). Fluorescence intensities in green, red and overlapping spectral regions were determined and analyzed by using an "apoptosis correlator" plugin operated in the Image J software.

Quantificiation of apoptosis by enzyme-linked immunosorbent assay (ELISA)

For apoptosis ELISA, the cells were processed in a cell death detection ELISA kit. The cells were incubated in lysis buffer for 30 min and centrifuged at 8000 rpm for 10 min. The supernatants were transferred into a streptavidin-coated microplate and incubated

with biotin-conjugated anti-histone and peroxidase-conjugated anti-DNA monoclonal antibodies for 2 h. After washing, ABTS substrate solution was added to each well for 15 min. Absorbance was measured at 405 and 490 nm. The specific enrichment of mono- and oligonucleosomes was calculated as enrichment factor (EF) = absorbance of RV-infected cells/absorbance of corresponding noninfected control cells.

Quantification of apoptosis by Annexin V/Propidium iodide (AN/PI) double labeling

For AN/PI double labeling, the cells were stained with FITC-labeled annexin V (AN-FITC) and propidium iodide (PI) according to the manufacturer's instructions. The fluorescence intensities of AN-FITC and PI were determined with a BD Bioscience flow cytometer (BD Biosciences, San Diego, CA, USA). The percentages of apoptotic cells were calculated by sorting the cells that were positive only for AN (early apoptotic stage) or for both AN and PI (late apoptotic or secondary necrotic stages).

Transmission electron microscopy

Samples were fixed in 0.1 M sodium cacodylate-buffered 2.5% glutaraldehyde solution (pH 7.4) for 2 h and then rinsed three times for 10 min each in 0.1 M sodium cacodylate buffer and 7.5% saccharose. The samples were post-fixed in 1% OsO4 solution for 1 h. After dehydration in an ethanol gradient (70%, 96% and 100% ethanol for 20 min each), samples were embedded in Durcupan ACM. Ultrathin sections were stained with uranyl acetate and lead citrate. Sections were examined in a Philips CM 10 microscope (Philips Electronic Instruments, Mahwah, NJ, USA) at 80 kV.

Western blot analysis

Cells were homogenized in CytoBuster lysis buffer, and the mixture was then centrifuged at 10000 *g* for 10 min to remove cell debris. Protein concentrations of cell lysates were determined by using the Bio-Rad protein assay. Supernatants were mixed with Laemmli's sample buffer and boiled for 3 min. Aliquots of the supernatants, containing 40-50 µg of total protein to detect actin, HSV gD, LC3B and Atg12–Atg5 conjugate were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose filters. Preblocked blots were reacted with antibodies against actin, HSV gD, LC3B and Atg5 for 4 h in PBS containing 0.05% Tween 20, 1% dried non-fat milk and 1% BSA. Blots were then incubated for 2 h with peroxidase-conjugated species-specific antibodies. Filters were developed by using a chemiluminescence detection system. The autoradiographs were scanned with a GS-800 densitometer (Bio-Rad, Hercules, CA, USA), and the relative band intensities were quantified by use of the ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

Statistical analysis

All values are expressed as mean ± standard deviation (SD). Student's unpaired t-test or the one-way ANOVA test with the Bonferroni post-test were used for pairwise multiple comparisons, and P values < 0.05 were considered statistically significant (GraphPad Prism 5, GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

I. The effect of RV on autophagy in the SIRC cell line

To elucidate how RV affects the cellular autophagic cascade, *(i)* the replication kinetics of RV, *(ii)* the levels of LC3B and the Atg12–Atg5 conjugate, *(iii)* the autophagic flux,

(iv) the subcellular localization of LC3B and *(v)* cytoplasmic acidification were measured in SIRC cells infected at an MOI of 5.

I./1. Replication of RV in the SIRC cell line

Indirect immunofluorescence assays to determine the kinetics of virus multiplication revealed positive staining for the E1 envelope glycoprotein of RV on days 1, 3 and 5 in 6.2, 42.8 and 69.1 % of the cells, respectively. These data indicate that the SIRC cell line is highly permissive for RV replication, and the infection proceeds in a slow and asynchronous way.

I./2. The effect of RV on the expression levels of autophagosomal marker proteins

To study the effects of RV on the basal autophagy, the levels of LC3B-I, LC3B-II and the Atg12–Atg5 conjugate were determined by Western blot analysis. The mock-infected cells displayed endogenous expression of both the lipidated and the non-lipidated forms of LC3B at the 0-day time point, and the LC3B-II levels increased continuously and dramatically throughout the 5-day culture period. In contrast, RV-infected cells exhibited only moderate increases in LC3B-II levels at 3 and 5 dpi. The endogenous expression of the Atg12–Atg5 conjugate in the mock-infected cells was slightly downregulated during the 5-day culture period, while RV infection triggered a pronounced decrease in the level of the Atg12–Atg5 conjugate. Thus, RV infection decreases the levels of LC3B-II and the Atg12–Atg5 conjugate.

I./3. The effect of RV on the autophagic flux

To investigate the autophagic flux in RV-infected cells, LC3B-II levels were measured under conditions where autophagosome degradation was blocked by bafilomycin A1, a pharmacological inhibitor of autophagosome-lysosome fusion and lysosomal hydrolase activity. As bafilomycin A1 exerted cytotoxic effects when it was present throughout the infection, the cultures were infected first and treated with bafilomycin A1 for a 24-h period just before the preparation of cell lysates. As expected, bafilomycin A1 elevated the level of LC3B-II as compared with the untreated mock-infected cells, indicating that this drug efficiently blocked autophagic flux under the experimental conditions used. Interestingly, the LC3B-II level of RV-infected cells incubated in the presence of bafilomycin A1 was considerably lower than that of the drug control. Thus, RV infection impedes the autophagic flux.

I./4. The effect of RV on autophagosome formation

To study the effects of RV on the intracellular localization of endogenous LC3B during basal and rapamycin-induced autophagy, indirect immunofluorescence assay was used. Both mock-infected and rapamycin-treated cells displayed patterns characterized by the combination of a diffuse, faint and punctate, bright LC3B staining. Accordingly, line scan fluorescence intensity analyses revealed numerous robust peaks, which were confluent or more separated in the mock-infected or rapamycin-treated cells, respectively. In contrast, RV-infected cells exhibited very faint cytoplasmic staining with only a few bright puncta, and the line scan profile consisted mainly of peaks of low height. Moreover, the intensity level of LC3B staining in RV-infected cells treated with rapamycin was much lower than that of the drug control. Thus, RV infection inhibits the intracellular redistribution of LC3B from the cytoplasm to autophagosomes. To further investigate the effects of RV on autophagosome formation, the abundance and size of LC3B-positive vesicles were also determined. The average numbers of LC3B-positive vesicles per cell in the mock-infected and rapamycin-treated cultures were 19.76 and 21.89, respectively. In contrast, the average numbers of puncta in RV-infected cultures were 12.49 and 12.81 in the absence or presence of rapamycin, respectively. Thus, RV infection triggers a significant decrease in the accumulation of autophagosomes. The

average Feret diameters of LC3B-positive vesicles in the mock-infected and rapamycin-treated cultures were 657 and 680 nm, respectively. Intriguingly, the average diameters of the puncta in the RV-infected cultures when cells were incubated with rapamycin or left untreated were 481 or 480 nm, respectively. Thus, RV infection elicits a significant decrease in the size of the autophagosomes. To determine the effects of RV infection on the formation of acidic vesicular organelles (AVOs), AO staining was used. In the mock-infected cultures, the orange nuclei were round in shape with a homogeneous inner structure, and the cytoplasm exhibited bright-red staining with a marked punctate structure. In the RV-infected cultures, the nuclei were irregular in shape and size, with a heterogeneous inner structure displaying a variable staining pattern, and the cytoplasm stained pale-red with faint puncta. Analysis of the fluorescence intensities in green, red and overlapping spectral regions revealed a reduction in red and an enhancement of orange fluorescence in response to RV infection. Thus, RV infection inhibits cytoplasmic acidification and blocks the development of AVOs.

I./5. The connection between autophagy and apoptosis in RV-infected cells

To study how rapamycin-induced autophagy modulates the infectious process of RV, we infected cells either directly or in the presence of rapamycin, and measured *(i)* the virus yields and *(ii)* the extent of apoptosis.

To determine the effects of rapamycin on the level of autophagy in mock- and RV-infected cells, LC3B expression was measured by Western blot analysis. Rapamycin triggered an increase in LC3B-II expression as compared with the mock-infected control. This result, corroborating the data obtained by using immunofluorescence analysis, indicates that this drug efficiently stimulated autophagy under the given experimental conditions, and the autophagic pathway controlled by the mTOR is intact in the SIRC cell line. The LC3B-II level of RV-infected cultures incubated in the presence of rapamycin was considerably lower than that of the drug control. Thus, RV inhibits rapamycin-induced autophagy. Plaque assays to measure virus yields at 5 dpi revealed that the titers of culture supernatants harvested from RV-infected cultures incubated in the absence or presence of rapamycin were on average 4.0×10^6 or 2.7×10^6 PFU/ml. Thus, rapamycin-mediated induction of autophagy inhibits RV multiplication. ELISA to quantify the extent of apoptotic DNA fragmentation at 5 dpi demonstrated low apoptotic levels in the mock-infected and rapamycin-treated cultures; the EFs measured in the absence or presence of rapamycin were 1 or 1.5, respectively. Cells infected with RV either directly or in the presence of rapamycin displayed highly increased apoptotic rates; the EFs measured in the absence or presence of rapamycin were 11.3 or 8.0. Interestingly, the apoptotic response of RV-infected cells in the absence of rapamycin was significantly higher than that of cells treated with this autophagy inducer. Thus, rapamycin-mediated induction of autophagy inhibits the apoptosis triggered by RV.

II. The effect of HSV-1 and HSV-2 on autophagy in the SIRC cell line

To elucidate how HSV-1 and HSV-2 affect the cellular autophagic cascade, *(i)* viral replication, *(ii)* the levels of LC3B and the Atg12–Atg5 conjugate, *(iii)* the autophagic flux, *(iv)* the subcellular localization of LC3B and *(v)* cytoplasmic acidification were measured in SIRC cells.

II./1. Replication of HSV-1 and HSV-2 in the SIRC cell line

The SIRC cell line was infected with either the KOS strain of HSV-1 or a wild-type HSV-2 strain at various multiplicities. Virus replication was measured by indirect immunofluorescence assay, Western blot and plaque titration at 24 hpi. Indirect

immunofluorescence assay revealed positive staining for gD in 97% and 91% of cells infected with HSV-1 or HSV-2, respectively. Western blot analysis demonstrated the presence of gD in cultures infected with HSV-1 or HSV-2 at MOIs of 0.1, 1 and 10. The production of progeny virus was determined by plaque titration of the culture supernatants taken from SIRC cells. Depending on the infectious dose, the level of HSV-1 production varied between 2.3 x 10^5 and 3.1 x 10^7 PFU/ml. The level of HSV-2 production varied between 6.8 x 10^4 and 3.7 x 10^7 PFU/ml. Together, these data demonstrate that the SIRC cell line is permissive for both HSV-1 and HSV-2 infection.

II./2. The effects of HSV-1 and HSV-2 on basal autophagy

To study the effects of HSV-1 and HSV-2 on the process of autophagy, SIRC cells were infected either with HSV-1 or HSV-2 at an MOI of 1 and analyzed by TEM and immunofluorescence assay at 24 hpi. TEM examination of the mock-infected culture revealed normal cell morphology. In contrast, there were numerous intracytoplasmic vacuoles in HSV-1-infected cells. These vacuolar structures contained amorphous materials, organelles or virion particles. The vacuoles were dispersed in the cytoplasm evenly. This finding corresponds to the TEM picture of excessive autophagy. This test has also provided experimental proof of ongoing viral replication in SIRC cells, as numerous assembled capsids and primarily enveloped virions were revealed in the nuclei of infected cells. Some fully matured cytoplasmic capsids and virions released from the cell in exocytotic vesicles were also detected. Indirect immunofluorescence assay to investigate the effect of HSV-1 on the intracellular localization of Atg5 and LC3B revealed diffuse, cytoplasmic staining patterns in the mock-infected cultures. In contrast, HSV-1 increased the intensity of staining and triggered translocation of Atg5 and LC3B from the cytoplasm to distinct puncta in 67% and 93% of cells, respectively. Together, these data demonstrate that HSV-1 triggers the formation of autophagosomes and alters the intracellular localization of Atg5 and LC3B.

II./3. The effects of HSV-1 and HSV-2 on the autophagic flux

To investigate the autophagic flux, bafilomycin A1 (BFLA) was used as a pharmacological tool to inhibit lysosomal degradative activity and to prevent fusion of autophagosomes with lysosomes. The cells were infected either with HSV-1 or HSV-2 at an MOI of 20, and incubated for 3 h. The cultures were then treated with BFLA for an additional 12 h period, and analyzed for viral yields, apoptosis and LC3B expression. Western blot analysis to quantify intracellular gD protein revealed two bands migrating near 52 and 56 kDa in HSV-1- or HSV-2-infected cells. BFLA inhibited the accumulation of this envelope glycoprotein in HSV-1- or HSV-2-infected cells by 12.3% and 42.5%, respectively. Plaque assay to quantify viral multiplication revealed that BFLA retarded replication of HSV-1 and HSV-2 by 52.6% and 13.6%, respectively. Thus, the strategy involving delayed addition of BFLA provided conditions when productive infection has occurred, though at diminished levels compared to infected cultures incubated in the absence of this autophagy inhibitor. Western blot analysis to measure the levels of the lipidated and non-lipidated forms of LC3B demonstrated that the mock-infected culture displayed endogenous expression both of LC3B-II and LC3B-I; the LC3B-II / LC3B-I ratio was 0.17 at 15 h of culturing. Cells infected with HSV-1 and HSV-2 displayed elevated LC3B-II and depressed LC3B-I levels; the LC3B-II / LC3B-I ratios were 0.82 and 0.56, respectively. This result demonstrates that HSV-1 and HSV-2 promote LC3B lipidation. BFLA highly elevated the level of LC3B-II, indicating that this drug efficiently blocked autophagy under the experimental conditions used, and the pathways promoting LC3B-II accumulation are intact in the SIRC cell line. In the presence of BFLA, HSV-1 and HSV-2 triggered slightly lower

increase in the level of LC3B-II than that observed in the corresponding drug control. Thus, both HSV-1 and HSV-2 decrease the autophagic flux to some extent.

II./4. The effects of HSV-1 and HSV-2 on the formation of acidic vesicular organelles

Acridine orange staining to detect AVOs revealed green cytoplasmic and nuclear staining in the mock-infected cells at 24 h of culturing. The nuclei of mock-infected cells were mostly round in shape. In contrast, cells infected with HSV-1 or HSV-2 at an MOI of 1 displayed green nuclear and strong red cytoplasmic fluorescence at 24 hpi, demonstrating that both HSV types promote cytoplasmic acidification. In HSV-1-infected cultures a few small syncytia, containing <5 nuclei, were observed, while HSV-2 infection resulted in the formation of numerous large multinucleated giant cells, which contained >50 nuclei. The nuclei of infected cells had markedly irregular shape, contained disorganized chromatin structures, and in some areas blurring, folding or complete disruption of the nuclear membrane was also observed. Thus, HSV-1 and HSV-2 trigger the development of AVOs.

II./5. The connection between autophagy and apoptosis in cells infected with HSV-1 or HSV-2

To investigate the cytopathogenicity of HSV-1 and HSV-2 in SIRC cultures, the cell death pattern of cultures infected at an MOI of 1 was determined by AN/PI double labeling at 24 hpi. Both HSV-1 and HSV-2 decreased the proportions of viable cells by 32.9% and 57.8%, respectively. The proportions of AN-single-positive (early apoptotic) and double positive (late apoptotic or secondary necrotic) cells in HSV-1-infected cultures were increased by 15.4% and 13.3%, respectively. The proportions of double-positive and PI single positive (necrotic) cells were increased by 36.5% and 23.8% in HSV-2-infected cultures, respectively. Together, these data reveal that both HSV-1 and HSV-2 trigger extensive cell demise. To investigate the potential connection between autophagy and apoptosis, the effect of the autophagy inhibitor BFLA was assessed. The cells were infected either with HSV-1 or HSV-2 at an MOI of 20, and incubated for 3 h. The cultures were then treated with BFLA for an additional 12 h period, and analyzed for apoptosis by ELISA. The assay revealed significantly higher apoptotic rates in HSV-1- and HSV-2-infected cells; 1 the EFs for HSV-1 and HSV-2 were 2.86 and 2.93, respectively. EFs for the HSV-1- and HSV-2-infected cells incubated in the presence of BFLA were 6.36 and 4.32 vs. 2.86 and 2.93 for the HSV-1- and HSV-2-infected cells incubated in the absence of drug, respectively. Thus, BFLA triggered a significant increase in the apoptotic responses of HSV-1- and HSV-2-infected cells as compared with the corresponding infected controls incubated in the absence of drug. Moreover, there was a significant difference between the apoptosis inducing activities of HSV-1 and HSV-2 in BFLA-treated cells. Together, these results demonstrate that inhibition of autophagy facilitates apoptosis induced by HSV-1 and HSV-2.

Discussion

I. The effect of RV on the process of autophagy

Positive-stranded RNA viruses, including several members of the *Picornaviridae*, *Flaviviridae* and *Togaviridae* families, have been shown to promote the generation and accumulation of autophagosomes in the infected cells. A great body of experimental evidence indicates that poliovirus, coxsackievirus B3, human rhinovirus 2, enterovirus 71, hepatitis C virus, dengue virus and Japanese encephalitis virus induce and actively exploit autophagy to promote their own replication. Our study has provided evidence that, unlike alphaviruses and several other positive-stranded RNA viruses, the To336 strain of RV inhibits autophagy. Initially, four distinct criteria of perturbed autophagy

were evaluated in RV-infected SIRC cells. As the phosphatidylethanolamine-conjugated form of LC3B and the Atg12–Atg5 complex are the best-characterized markers of autophagic activity, the levels of these components of autophagy were measured by Western blot analysis. The data revealed that RV infection decreased LC3B-II accumulation in comparison with the corresponding mock-infected control, and this was accompanied by a decrease in the Atg12–Atg5 conjugate level at 3 and 5 dpi. The autophagic flux was also assessed by monitoring the LC3B-II levels in mock- and RV-infected cultures incubated in the presence of the vacuolar H^+-ATPase inhibitor BFLA. In the presence of BFLA, the LC3B-II level of RV-infected cells was substantially lower than that seen in the drug control, demonstrating that the autophagic flux was markedly reduced by this virus. Additionally, high-resolution confocal imaging was performed to evaluate autophagosome formation. These experiments revealed that RV suppressed the intensity level of LC3B staining, and triggered significant decreases in both the number and the average surface area of the autophagosomes, suggesting that this virus inhibits the development of autophagosomes. Finally, the effect of RV on AVO formation was determined by using AO staining. The results demonstrated that RV infection inhibits cytoplasmic acidification and impedes the development of AVOs. Previous interesting observations have clearly demonstrated that an active autophagy flux requires the covalent attachment of Atg12 to Atg5, and in the absence of this linkage, autophagy cannot proceed. Further studies have also revealed that defects in LC3 function lead to the failure of autophagosome closure. In view of these findings, our present results are generally consistent with a scenario in which an RV-induced decrease in the level of the Atg12–Atg5 conjugate impairs LC3B lipidation, which in turn may perturb autophagosome formation in RV-infected cells. Another interesting earlier study found that RV increases the phosphorylation of Akt and its downstream effector molecules, phosphoprotein 70 ribosomal protein S6 kinase (p70S6K) and glycogen synthase kinase 3β (GSK3β). Moreover, the inhibition of PI3K by using LY294002 resulted in increases in the speed and magnitude of RV-induced apoptosis in the RK-13 cell line. It is now widely accepted that Akt activates mTOR complex 1 (mTORC1) by inhibiting two known cellular mTORC1 inhibitors [tuberous sclerosis complex 2 (TSC2) and the proline-rich Akt substrate of 40 kDa (PRAS40)], thereby stimulating cap-dependent translation, promoting cell survival and inhibiting autophagy. Thus, it is reasonable to infer that activation of the PI3K/Akt/mTOR axis of signaling may play some role in the inhibition of autophagy in RV-infected cells.

Since some viruses benefit from autophagy, whereas others are harmed, next the effect of autophagy induction on RV multiplication was evaluated. Viral titers of culture supernatants harvested from RV-infected cultures incubated in the absence or presence of rapamycin were measured by plaque assay. The significantly reduced viral yield seen in the presence of rapamycin suggested that autophagy can control RV multiplication to some extent. The premature death of infected cells by way of apoptosis can function as an important antiviral mechanism, especially in the case of a virus which is characterized by a slow replication kinetics, such as RV. As autophagy was shown to operate as a facilitator, cooperator or antagonist of apoptosis, depending on the nature of the environmental cue and the cellular context, the effect of autophagy induction on the apoptotic responses of infected cells was evaluated. The experiments clearly indicated that RV triggered apoptosis in SIRC cells, and the extent of cell demise induced by this virus was significantly reduced by the induction of autophagy. Thus, these data lend support to the view that autophagy is an efficient antiviral mechanism that does not involve apoptosis induction in RV-infected cells.

Overall, these results suggest that RV employs a strategy to cope with autophagy that is totally different from that evolved by other positive-stranded RNA viruses, and there is a considerable heterogeneity among the members of the *Togaviridae* family in terms of their effects on the cellular autophagic cascade. Besides its cytoprotective effects, autophagy furnishes an important antiviral mechanism, inhibition of which may reorchestrate the intracellular environment so as to better serve the unique requirements of RV replication. Moreover, the RV-mediated inhibition of autophagy may be implicated in the pathogenesis of the congenital rubella syndrome, as functional autophagic machinery is of pivotal importance for normal fetal development.

II. The effects of HSV-1 and HSV-2 on the process of autophagy

DNA viruses, including several members of the *Herpesviridae* family have been shown to modulate the process of autophagy in the infected cells. Previous studies have demonstrated that the effect of HSV-1 on autophagy is cell-type-specific. While HSV-1 activates the STING (stimulator of interferon genes)-dependent autophagy pathway in murine macrophages and dendritic cells, neuroblastoma cells respond to HSV-1 infection with an abortive autophagy, and the anti-autophagic effects of ICP34.5 and Us11 proteins dominate in fibroblasts and primary murine neurons. Further interesting studies have revealed that varicella-zoster virus, lacking genes that encod anti-autophagic proteins, activates autophagy *in vitro* and in zoster vesicles. A great body of experimental evidence also indicates that human cytomegalovirus (CMV) enhances some specific steps in the autophagic cascade, while it inhibits others. During the first hours of infection, CMV stimulates autophagy in the absence of *de novo* synthesis of viral proteins, and thereby leads to increased expression and lipidation of LC3B. However, at later stages of infection, CMV blocks the autophagic cascade via stimulating mTOR and inhibiting PKR, and thereby triggers a dramatic decrease in the number of autophagosomes. Other remarkable studies demonstrate that Epstein-Barr virus (EBV) modulates the unfolded protein response (UPR) and autophagy in a coordinated fashion so as to foster the latent form of its life cycle. The latent membrane protein 1 (LMP-1) activates the signaling pathway coupled to CD40, essential for proliferation of EBV-infected B lymphocytes. LMP-1 triggers UPR dose dependently, which in turn evokes (*i*) an increase in the expression of LMP-1 protein of EBV, (*ii*) a general block in cellular protein synthesis, (*iii*) induction of apoptotic cell demise and (*iv*) stimulation of the autophagic cascade. Once activated, autophagy facilitates degradation of LMP-1, and thereby promotes reconciliation of the diverse cellular response to restore the physiological state of cells. Taken together, the available data suggest that modulation of autophagy by viruses belonging to the *Herpesviridae* family plays important role in the infectious process, and functions as part of their immune escape strategies. However, the precise biological importance of autophagy in the pathogenic mechanisms of infections caused by these viruses is not yet fully understood.

Our study has provided evidence that HSV-1 facilitates the accumulation of autophagosomes, as well as the recruitment of LC3B and Atg5 to autophagic vacuoles in the SIRC cell line. Our further experiments have also shown that both HSV-1 and HSV-2 trigger LC3B lipidation, and increase cytoplasmic acidification, without enhancing the autophagic flux. Together, these results demonstrate that autophagy is induced by both HSV-1 and HSV-2 in spite of their effective anti-autophagic armamentarium, which possibly impedes the process considerably via inhibiting the fusion of autophagosomes with lysosomes.

Apoptosis has already been implicated in the maintenance of ocular surface integrity both in physiological and pathological conditions. Compelling evidence indicated that

apoptosis is instrumental in glaucoma, retinitis pigmentosa, cataract, retinoblastoma, retinal ischemia, diabetic retinopathy and several eye infections caused by viruses, bacteria, fungi and some protozoans. Further remarkable studies focusing on the function of corneal keratocytes in the maintenance of visual integrity have clearly demonstrated that keratocytes, located in the corneal stroma as a highly interconnected cellular network, undergo apoptotic cell demise immediately after infections. The apoptotic response of keratocytes was shown to operate as an important antiviral mechanism by inhibiting the spread of viruses to deeper structures of the eye, and minimizes the inflammation that accompanies corneal injury. In full accord with these findings our data have corroborated that both HSV-1 and HSV-2 trigger apoptosis in corneal cells.

Recent findings demonstrated that the apoptotic, autophagic and necrotic pathways are interconnected and subject to coordinated regulation. Autophagy can operate as antagonist, facilitator or cooperator of apoptosis depending on the nature of stressor and cellular context. It has also been shown that HSV-1 elicits an mTOR-independent pathway of autophagy, which in turn prolongs survival of U251 glioma cells by counteracting the coinciding apoptotic response. Thus, next we have asked whether autophagy has the potential to modulate apoptotic demise during the course of HSV-1 and HSV-2 infection. Our results have revealed that apoptotic cell demise induced by both HSV-1 and HSV-2 is markedly enhanced by inhibition of autophagy indicating that autophagy acts as antagonist of apoptosis in HSV infected SIRC cells. Induction of autophagy by HSV-1 and HSV-2 may represent a viral strategy to provide sufficient time for replication via inhibiting untimely apoptotic demise. These data corroborate previous findings obtained with HSV-1-infected U251 glioma cultures, and extend the spectrum of cells in which HSV mediated induction of autophagy alleviates apoptosis. Under circumstances when apoptosis is blocked, viral replication can proceed in an undisturbed fashion, and the inevitable demise of HSV-infected cells may preferably occur by way of necrosis. This type of cell death may trigger a strong proinflammatory response and thereby it may contribute to the development of characteristic clinical symptoms seen in keratitis patients. Together, these results may bear on keratitis, since dysregulation of keratocyte apoptosis may account for the structural and functional damage of cornea occurring during the course of HSV infection. Moreover, our further observation demonstrating a more remarkable anti-apoptotic effect of autophagy in cultures infected with HSV-1 than in HSV-2-infected cells suggests that the mechanism of corneal cytopathogenicity evoked by the two HSV types might involve some different molecular mechanisms.

A better understanding of intrinsic autophagy evolving during the course of herpes simplex keratitis and other infections caused by HSV-1 and HSV-2 may provide useful knowledge for the development of novel therapeutic modalities acting through autophagy modulation. A recent interesting study revealed that rapamycin used topically in a mouse model of HSK suppressed inflammation and neovascularization. In view of our present findings obtained by using an *in vitro* SIRC corneal cell model system, it is reasonable to infer that the beneficial therapeutic efficiency of autophagy inducers in herpetic keratitis may partly be due to their ability to reduce tissue damage via counteracting apoptotic demise of infected cells.

Overall, our study demonstrates that HSV-1 and HSV-2 affect autophagy and apoptosis in a coordinated fashion, and autophagy antagonizes apoptosis in HSV-infected cells. Together these data implicate autophagy in the pathogenic mechanisms of herpetic keratitis.

The following of our results are considered novel:

1. RV inhibits autophagy in the SIRC cell line.
2. RV blocks the autophagic flux.
3. RV inhibits the development of autophagosomes.
4. RV inhibits cytoplasmic acidification.
5. Activation of autophagy inhibits RV-mediated induction of apoptosis.
6. HSV-1 and HSV-2 stimulate autophagy, without increasing the autophagic flux in the SIRC cell line.
7. HSV-1 facilitates the accumulation of autophagosomes.
8. HSV-1 and HSV-2 increase cytoplasmic acidification.
9. Inhibition of autophagy enhances apoptosis triggered by HSV-1 or HSV-2.

SUMMARY

Autophagy and apoptosis function as important early cellular defense mechanisms in infections and other diseases. The outcome of an infection is determined by a complex interplay between the pathogenic microorganism and these intracellular pathways. To better understand the cytopathogenicity of RV, HSV-1 and HSV-2, we have studied the effect of these viruses on the autophagic and apoptotic processes in the SIRC corneal cell line.

Infection with the To336 strain of RV lowered the levels of LC3B-II and the Atg12–Atg5 conjugate, inhibited the autophagic flux, suppressed the intracellular redistribution of LC3B, decreased both the average number and the size of autophagosomes per cell, and impeded the formation of acidic vesicular organelles. Induction of autophagy by using rapamycin decreased both the viral yields and the apoptotic rates of infected cultures. Thus, besides its cytoprotective effects, autophagy furnishes an important anti-viral mechanism, inhibition of which may reorchestrate intracellular environment so as to better serve the unique requirements of RV replication. Together, these data suggest that RV utilizes a totally different strategy to cope with autophagy than that evolved by other positive-stranded RNA viruses, and there is considerable heterogeneity among the members of the *Togaviridae* family in terms of their effects on the cellular autophagic cascade. The RV-mediated inhibition of autophagy may be implicated in the pathogenesis of the congenital rubella syndrome, as functional autophagic machinery is of pivotal importance for normal fetal development.

Infection with the KOS strain of HSV-1 and a wild-type strain of HSV-2 enhanced autophagosome formation, triggered cytoplasmic acidification, increased LC3B lipidation and elevated the ratio of apoptotic cells. The autophagy inhibitor BFLA triggered a significant increase in the apoptotic responses of HSV-1- and HSV-2-infected cells. Thus, both HSV types affect autophagy and apoptosis in a coordinated fashion, and autophagy plays cytoprotective role in HSV-infected cells via antagonizing apoptosis. Induction of autophagy by HSV-1 and HSV-2 may represent a viral strategy to provide sufficient time for replication via inhibiting untimely apoptotic demise. Together these data implicate autophagy in the pathogenic mechanism of herpetic keratitis.

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