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

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CONTENTS

LIST OF PUBLICATIONS

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I. K. Pásztor, L. Orosz, Gy. Seprényi, K. Megyeri, Rubella virus perturbs autophagy. Med Microbiol Immun, **2014**, DOI: 10.1007/s00430-014-0340-7
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III. K. Megyeri, L. Orosz, B. Kormos, K. Pásztor, Gy. Seprényi, Y. Mándi, Zs. Bata-Csörgő, L. Kemény, The herpes simplex virus-induced demise of keratinocytes is associated with a dysregulated pattern of p63 expression. Microb Infect, **2009**, 11: 785-794
IF: 2.51

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ABBREVIATIONS

ADP	Adenosine diphosphate
AN/PI	Annexin V/Propidium-iodide
AO	Acridine orange
Asc	Apoptosis-associated speck like protein containing a caspase activation and recruitment domain
Atg	Autophagy related gene
ATP	Adenosine triphosphate
AVOs	Acidic vesicular organelles
Bcl-2	B-cell lymphoma 2
BFLA	Bafilomycin A1
BHK21	Baby hamster kidney fibroblast
BRCA1	Breast cancer type 1 susceptibility protein
BSA	Bovine serum albumin
C	Capsid protein
CK	Citron-K kinase
CRS	Congenital Rubella Syndrome
DAMPS	Damage-associated molecular patterns
DFCP1	Double FYVE-containing protein 1
DISC	Death inducing signaling complex
DNA	Deoxyribonucleic acid
dpi	Day(s) postinfection
EF	Enrichment factor
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FCS	Fetal calf serum
FITC	Fluorescein isothyocianate
gB	Glycoprotein B
gC	Glycoprotein C
gD	Glycoprotein D
gE	Glycoprotein E
gG	Glycoprotein G

gI	Glycoprotein I
gL	Glycoprotein L
gM	Glycoprotein M
GSK3β	Glycogene synthase kinase 3 β
hpi	Hour(s) postinfection
HSV	Herpes simplex virus
ICP	Infected cell protein
IE	Immediate early
IL	Interleukin
IRE1α/XBP1	Inositol-requiring enzyme 1α/X boxbinding protein 1
kDa	Kilodalton
LC3	Microtubule-associated protein 1 light chain 3
LIR	LC3 interacting region
LMP-1	Latent membrane protein 1
mM	Millimolar
MMR	Measles, Mumps, Rubella vaccine
MOI	Multiplicity of infection
MOMP	Mitochondrial outer membrane permeabilization
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex 1
NAD^+	Nicotinamide adenine dinucleotide
NBR1	Neighbor of BRCA1 gene 1 protein
NDP52	Nuclear domain 10 protein 52
ORF	Open reading frame
p53	Tumor suppressor p53
PARP1	Poly(ADP-ribose) polymerase 1
PBS	Phosphate buffered saline
PFU	Plaque forming unit
PI3K	Phosphatidylinositol 3-kinase
PKR/eIF2α	Double stranded RNA-dependent protein kinase/eukaryotic translation initiation factor 2 α
pRB	Retinoblastoma tumor suppressor protein
Rapa	Rapamycin
RER	Rough endoplasmic reticulum

RipK	Receptor-interacting serine/threonine protein kinase
RK13	Rabbit kidney epithelial cells
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RV	Rubella virus
SIRC	Staatens Seruminstitute Rabbit Cornea cell line
SMURF1	p62/SQSTM1-Sma and Mad-related family ubiquitin regulatory factor 1
SQSTM1	Sequestosome-1
STING	Stimulator of interferon genes
TEM	Transmission electronmicroscopy
TK	Thymidine kinase
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
U_L	Long unique region
ULK1	Autophagy related gene 1 (Atg1) homolog
U_S	Short unique region
VP	Viral protein

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.

I. Major cell death subroutines

Apoptosis is a major form of cell death characterized by specific morphological signs, such as cell shrinkage, membrane blebbing and chromatin condensation. Two main evolutionarily conserved protein families are involved in apoptosis, namely the Bcl-2 family member proteins, which control mitochondrial integrity (1, 2) and the cysteinyl aspartate-specific proteases or caspases, which mediate the execution phase of apoptosis. (3) Apoptosis is classified into two cell death subroutines: *(i)* the extrinsic and *(ii)* the intrinsic apoptosis. (4-7) Extrinsic apoptosis is a caspase-dependent cell death pathway that is induced by binding of lethal ligands to death receptors, such as Fas/CD95, tumor necrosis factor (TNF) α receptor 1 and TNF-related apoptosis inducing ligand receptor. (8, 9) Ligand binding triggers the assembly of a supramolecular complex termed death inducing signaling complex (DISC) at the cytoplasmic tail of the receptor. (10, 11) DISC functions as a platform that regulates the activation of caspase-8 or -10. (11-13) Intrinsic apoptosis is triggered by various intracellular stress conditions, including DNA damage, oxidative stress, cytosolic Ca^{2+} overload, and accumulation of unfolded proteins in the cytoplasm. (4) Signaling cascades that trigger intrinsic apoptosis converge in mitochondria and elicit mitochondrial outer membrane permeabilization (MOMP). MOMP leads to bioenergetic and metabolic catastrophe via several mechanisms, including disruption of the mitochondrial transmembrane potential, inhibition of the respiratory chain, and release of toxic proteins from the mitochondrial intermembrane space into the cytosol. (14-17)

Anoikis is an ancient Greek term meaning the ‘state of being homeless’. (18, 19) Anoikis is a particular apoptotic cell death that occurs in adherent cells in response to forced detachment from the matrix. (20, 21) This form of cell demise can become activated by the lack of β1-integrin, downregulation of epidermal growth factor receptor

expression, inhibition of extracellular-regulated kinase 1 signaling, and overexpression of the Bcl-2-interacting mediator of cell death (Bim) protein. (18, 21) The cell death program of anoikis is driven by the molecular machinery of intrinsic apoptosis. (21, 22) Death of normal cells by way of anoikis inhibits dysplastic growth by preventing reattachment of displaced cells to new matrices. (20) Resistance to anoikis of transformed epithelial cells facilitates invasiveness and increases metastatic potential. (23-25)

Necrosis is an accidental form of cell death that can be caused by external factors, such as infection, toxins, cancer, infarction, poisons, ROS (Reactive Oxygen Species), inflammation or trauma. (26) Necrotic cell death is characterized by cytoplasmic and organelle swelling, followed by the loss of cell membrane integrity and release of the cellular contents into the surrounding extracellular space.

Necrosis can also occur in a regulated manner. (27, 28) Regulated necrosis or necroptosis can be triggered by members of the TNF family, Toll-like receptors (TLR3 and TLR4) and DNA and/or RNA sensors. (27) Necroptosis is characterized by the lack of specific apoptotic markers, such as caspase activation, while it depends on the activation of receptor-interacting protein 1 (RipK1) and RipK3 kinases. (29, 30) Necroptosis leads to rapid plasma membrane permeabilization, release of cell contents, and exposure of damage-associated molecular patterns (DAMPs). It has an important role in T cell homeostasis, and during viral infections necroptosis works as a back-up mechanism to eliminate the pathogen. (28)

Parthanatos is a particular case of regulated necrosis. Parthanatic cell demise characteristically depends on early activation of poly(ADP-ribose) polymerase 1 (PARP1). (31) PARP1 overactivation triggers depletion of NAD^+ and ATP, disruption of the mitochondrial transmembrane potential, and release of apoptosis-inducing factor, which in turn leads to chromatinolysis. (32, 33)

Pyroptosis is a more recently recognized form of regulated cell death with biochemical properties distinct from necrosis and apoptosis. (26, 34, 35) This cell death subroutine has initially been described in some infections caused by bacteria, such as *Salmonella Typhimurium*, *Shigella flexneri*, *Francisella tularensis*, *Listeria monocytogenes and Pseudomonas aeruginosa*. (35-39) Later, several other pyroptosis inducers have also been identified. (40, 41) The central mediator in the molecular mechanism of pyroptosis is caspase-1, which is activated in the cytosol by the inflammasome or pyroptosome. (35, 42) The inflammasome is a multiprotein complex composed of Nod-like receptors,

the adaptor protein Asc (apoptosis-associated speck-like protein containing a caspase activation and recruitment domain) and caspase-1. The pyroptosome is a supramolecular complex of Asc dimers. (42, 43) Activated caspase-1 induces the formation of ion-permeable pores with a diameter of 1.1 - 2.4 nm in the plasma membrane, and causes cell swelling and lysis. (44) In addition, caspase-1 activation causes the cleavage of some inflammatory cytokines such as pro-interleukin-1β (IL-1 β) and pro-IL-18 and thereby initiates an inflammatory response. (45)

Autophagy is an essential catabolic process that maintains cellular integrity by degrading cytoplasmic constituents and organelles. (46-48) It is highly activated by several pathological conditions, including nutrient starvation, oxidative stress, inflammation and infections. (49, 50) Autophagy can deliver sequestered cytoplasmic content, damaged proteins, organelles or pathogens to the lysosomal compartment using a double-membraned autophagosomal vacuole as a carrying vehicle. (47, 51-53) Under conditions of mild stress, autophagy plays a cytoprotective role by eliminating pathogenic microorganisms and dysfunctional organelles. In contrast, under conditions leading to massive cell damage, excessive autophagy possesses destructive power culminating in cell demise. (54-56) Autophagic cell demise is a cell death subroutine that can be blocked by pharmacological or genetic inhibition of the autophagic process. (57)

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. (46, 58, 59)

In chaperone-mediated autophagy, unfolded proteins are translocated directly across the limiting membrane of the lysosome. (60, 61)

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. (47, 51-53) Macroautophagy is induced by nutrient deprivation, cellular stress, infections or rapamycin treatment via complex signaling pathways. (53, 62, 63) The major regulator of macroautophagy is the mTOR (mammalian target of rapamycin) protein. (64, 65) The mTOR senses the nutrient

supply, energy status and growth factors, and inhibits the induction of autophagy. (64) Environmental stressors, starvation or rapamycin treatment inhibit the mTOR, and thereby trigger an increase in autophagic activity. (64, 65) 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. (66, 67) 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. (53, 56) 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. (68, 69) Accumulation of the lipidated form of LC3 is essential for the expansion and closure of the autophagosome membrane. (68-70) 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. (68, 70) 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. (71-73) The adaptors bind to LC3 via a domain known as LIR (LC3 interacting region), and deliver their substrates to autophagosomes. (74)

Autophagy plays important roles in numerous physiological processes and pathological conditions, including infections. (46, 75, 76) 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. (77) 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. (78-80)

II. Rubella virus and its interaction with the infected cells

Rubella virus (RV), the sole member of the *Rubivirus* genus in the *Togaviridae* family, can cause postnatal and congenital infections. Postnatal RV infection, known as rubella disease or German measles, is one of the five classic childhood diseases. By disturbing the ontogeny of fetal organs, congenital RV infection may lead to serious developmental abnormalities, collectively termed the congenital rubella syndrome (CRS). (81)

Structure and replication of RV

RV is an enveloped virus with a positive-sense, single-stranded RNA genome. (81) RV virions are spherical particles with a diameter of 55 - 90 nm. They have an electron-dense nucleocapsid core surrounded by a host-derived envelope. The RV nucleocapsid has icosahedral symmetry. The genome of RV contains about 70% guanine and cytosine, the highest of all sequenced RNA viruses. (82) The genome contains two open reading frames (ORFs). (81, 82) The 5' ORF encodes the two non-structural proteins (p150 and p90). (82-86) 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. (82, 84) The 3' ORF encodes the structural proteins, including the capsid (C) protein, and also the E1 and E2 envelope glycoproteins. (82)

The most characteristic feature of RV is that its intracellular life cycle is closely connected to the endomembrane system. RV enters the cells by using the endocytic pathway, and as early endosomes mature into late endosomes, acidification triggers the fusion of the RV envelope to the endosomal membrane and the uncoating of the C protein. (87-89) RV genomic RNA released into the cytoplasm serves as template for the synthesis of the non-structural proteins and a 40S negative-sense RNA strand. (90) The 40S negative-polarity RNA functions as a template for the genomic RNA and a 24S

sub-genomic RNA encoding the structural proteins. (85, 90) The non-structural proteins and replicating RNA remain in close association with the endosome, and stimulate the formation of spherule-like membrane invaginations intruding into the lumen of the endosome. (87) The nascent spherules remain attached to the endosomal membrane via a neck structure, and provide a protected environment for viral RNA replication, and for the synthesis of the non-structural proteins. (84, 85) The 24S subgenomic RNA is transported to the rough endoplasmic reticulum (RER), and the translated polyprotein precursor is processed by a cellular signalase, leading to the formation of the C, E1 and E2 structural proteins. (84, 91) The E1 and E2 glycoproteins then become embedded into the ER, and the C protein likewise remains membrane-associated. (91, 92) After being transported through the Golgi apparatus, the C protein reaches the modified endosome, and assembles with the newly synthesized genomic RNA expelled from the invaginations. As infection proceeds, the RV-modified endosome attracts several intracellular organelles, such as the RER, Golgi apparatus and mitochondria, together constituting the replication complex, also termed the RV factory. (87, 93, 94) The nucleocapsid of RV then matures via budding through the intracellular membrane, including the Golgi complex, ER and cytoplasmic vacuoles, or occasionally at the plasma membrane. (92, 94, 95) Together, these findings underscore the ability of RV to harness the endomembrane compartment in order to provide optimal conditions for almost every step of its replication.

The effect of RV on cell death

While RV establishes persistent, noncytocidal infection in many cell types, it can also trigger apoptotic cell death in some cell lines, such as the BHK21, Vero and RK13. (96-101) The ability of RV to induce apoptosis varies considerably with cell type and virus strain. (90) The non-structural proteins and C protein may be required for apoptosis induction. (96, 98) The non-structural protein p90 was shown to interact with some cellular proteins, such as the retinoblastoma tumor suppressor protein (pRb) and citron-K kinase (CK). (102, 103) The RV p90-pRb and RV p90-CK interactions may interfere with the physiological functions of pRb and CK. pRb is a crucial regulator of cell division. (104) Thus, the functional perturbation of pRb may dysregulate the G1-S phase transition of cell cycle progression, which in turn may lead to the activation of p53 and induction of the intrinsic pathway of apoptosis. (105) Indeed, previous *in vitro*

studies revealed a dramatic increase in p53 level, and demonstrated that a dominant-negative p53 mutant conferred partial protection from RV-induced apoptosis. (100) CK is a downstream target of the cellular Rho family small GTPases. The Rho proteins are important regulators of cytoplasmic formation of actin structures during cytokinesis. (106) Thus, the functional perturbation of CK may block cell cycle progression in the S-M phase transition, which in turn may lead to the generation of a subpopulation of cells with tetraploid nuclei. (106) RV C protein is an essential structural component of the virions, and a key factor in virus–host interactions. (107) Transfection studies have revealed that the C protein by itself can trigger apoptotic cell death. (96, 98, 99) Interaction of C protein with p32 mitochondrial protein regulating the opening of the permeability transition pore may play an important role in apoptosis induction. (108) Together, these results indicate that RV activates multiple pathways of apoptosis. (98-103) However, the effect of this virus on other cell death subroutines has not yet been investigated.

Pathogenesis and clinical syndromes

The infection caused by RV in early childhood or adult life is usually mild, self-limiting, and often asymptomatic. The infection is contracted via the respiratory route. RV replicates in the nasopharynx and lungs first, then spreads to the lymph nodes, and appears in the blood. The resulting viremia transfers the virus to various tissues and the skin. Clinically apparent postnatal rubella is characterized by maculopapular rash, lymphadenopathy and fever. (81, 82) The rash is the most prominent feature and exists in most of the cases. It begins on the face, and spreads in centripetal fashion. Infection in adults can be more severe, and can occasionally be complicated by more significant symptoms, such as arthropathy, thrombocytopenia and postinfectious encephalopathy. (82, 109, 110)

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. The embryo is at great risk until the 20^{th} week of pregnancy. (90, 111, 112) By disturbing the ontogeny of fetal organs, congenital RV infection may lead to serious developmental abnormalities. (90, 111) 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. (90) Although the teratogenic effect of this virus is not yet fully understood, numerous noteworthy studies suggest that the clues lie in the unusual features and unique cellular effects of RV replication. (90, 113-116)

No treatment is available for rubella. (117) The best means of preventing rubella is vaccination with a triple vaccine containing live, attenuated measles, mumps and rubella viruses (MMR). (81)

III. Herpes simplex viruses and their interaction with the infected cells

Herpes simplex viruses-1 and -2 (HSV-1 and -2) belong to the *Herpesviridae* family, commonly cause postnatal infections by invading the body through the skin and mucous membranes of the orofacial or genital regions. (118) HSV can also be contracted *in utero*, at the time of delivery or postnatally, leading to neonatal infection. (118)

Structure and replication of HSV-1 and HSV-2

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. (118, 119) The capsid is made up of four viral proteins, VP5 (gene U_L19), VP26 (gene U_L35), VP23 (gene U_L18), and VP19C (gene U_L38). The capsid also contains U_L6 protein, and VP24 protease (gene U_L26) required for the proteolytic processing of scaffold proteins. (118, 120) The space between the envelope and the capsid is largely unstructured, and it is composed of at least 20 viral proteins. The major proteins constituting the tegument are: *(i)* the virion host shutoff factor (gene U_L41), *(ii)* VP16 or α-trans-inducing factor, responsible for the transactivation of immediate early (IE) genes (gene U_L48), *(iii)* VP22 (gene U_L49), *(iv)* a very large tegument protein (VP1-2; gene U_L36) and *(v)* UL13 protein kinase. (121,122) The envelope consists of a lipid bilayer with approximately 11 different viral glycoproteins embedded in it. The virion envelope glycoproteins are gB (VP7 and VP8.5), gC (VP8; gene U_L44), gD (VP17 and VP18; gene U_S6), gE (VP12.3

and VP12.6; gene U_S8), gG (gene U_S4), gH (gene U_L22), gI (gene U_S7), gL (gene U_L1), and gM (gene U_L11). (118)

The envelope glycoproteins gC and gD play important role in the adsorption of HSV virion to cell surface receptors. gC attaches to heparan sulfate, while gD binds to herpesvirus entry mediator, nectin-1 and 3-O sulfated heparan sulfate. (123, 124) These interactions bring the viral envelope and the cell membrane into close proximity, and thereby allow the binding of gH, gL and gB to their corresponding receptors. (125, 126) After the fusion of the viral envelope with the plasma membrane, the capsid enters the cytoplasm and translocates to the nucleus, where transcription, replication of viral DNA and capsid assembly take place. (118, 127, 128) 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. (129, 130) During latency no viral particles are formed, only the latency-associated transcript is expressed, and the viral genome is maintained in an episomal state. (131-133) From this cellular reservoir, reactivation may occur. (130, 133) In other cell types, viral DNA remains linear, and a productive replication cycle ensues. (128) Initially, IE or α genes are transcribed, and their protein products transactivate the early or β and late or γ genes. (118, 134) By definition, early genes are those viral genes, which are expressed in the presence of IE proteins before the onset of DNA replication. (118, 127, 128) The products of early genes are primarily implicated in the replication of HSV DNA. (118) The late genes encode structural components of the HSV virion. (118) 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. (135) Following virion maturation, tegumented capsids gain their envelope by budding through the Golgi apparatus, and then released from the cell by reverse endocytosis. (136)

The effect of HSV-1 and HSV-2 on cell death

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. (118, 137) 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. (138-142) Moreover, both HSV-1 and HSV-2 have the capability to modulate the process of autophagy. (140-142)

Activation of the necrotic cell death subroutine has been revealed in several infections caused by HSV-1 and HSV-2, including encephalitis, acute retinal necrosis and hepatitis. (143, 144) The mechanism of necrotic tissue damage is complex and involves a direct cytopathic effect of HSV and indirect, immune-mediated events.

HSV-1 and HSV-2 are also capable of modulating the apoptotic cell death pathways. Early in infection with HSV-1 or HSV-2, the appearance of viral IE mRNAs activates the cellular apoptotic machinery. (132) However, at 3 to 6 hours postinfection (hpi), several anti-apoptotic HSV proteins (ICP4, ICP22, ICP17, ICP34.5, US3, US5, US6, UL14 and ICP10 of HSV-2), as well as the latency-associated transcript inhibit apoptotic demise, and maintain cell survival until the replication cycle is completed. The time period while anti-apoptotic HSV proteins are synthesized is termed “apoptosis prevention window”. (145) In this phase of infection, the intricate balance of pro- and anti-apoptotic factors is strongly biased by the indirect influence of powerful cellular defense pathways, such as the DNA damage response pathway or the interferon system. (146) Finally, HSV multiplication leads to the inevitable death of host cells, at least in part via apoptotic mechanisms. Apoptosis fulfils dual functions in the pathogenic mechanism of diseases caused by HSV-1 and HSV-2; it contributes to tissue damage and exerts antiviral effect by inhibiting viral spread at the expense of killing infected cells.

HSV-1 and HSV-2 may also affect the autophagic cascade. (147, 148) At the early stage of infection, HSV-1 provokes autophagy. (149) However, some viral proteins (ICP34.5 and US11) synthesized during the subsequent phases of productive infection function as powerful inhibitors of the autophagic process. (147) ICP34.5 of HSV-1 antagonizes autophagy by binding Beclin-1, and by blocking the double-stranded RNA-dependent protein kinase/eukaryotic translation initiation factor 2α (PKR/eIF2α) pathway. (149, 150, 151) The HSV-1 US11 protein exerts anti-autophagic activity via its direct interacion with PKR. (148) These interesting observations have shed light on the importance of autophagy in infections caused by HSV-1, as well as successfully uncovered the underlying molecular mechanisms in some experimental systems. At present, much less is known about the effects of wild-type HSV-2 on autophagy.

Pathogenesis and clinical syndromes

HSV-1 and HSV-2 invade the body through the skin and the mucous membranes of the orofacial or genital regions. During initial replication in epithelial tissues, HSV-1 and HSV-2 infect the sensory and autonomic nerve endings, and the nucleocapsids are transported intraaxonally to the nerve cell bodies in ganglia. The trigeminal ganglia are infected during the course of orofacial manifestations, while the sacral ganglia are more commonly affected in genital diseases. Viral replication takes place in neural tissues during primary infections, and eventually, a lifelong latency is established. The primary lesions usually heal completely. However, in response to local or systemic stressors, activation of productive viral multiplication may occur subsequently. HSV virions are then transported along the neuronal axons, back to peripheral tissues to cause recurrent lesions. This process is termed reactivation. (118, 152)

HSV-1 and HSV-2 can cause a variety of mild and severe diseases, including herpes simplex labialis, ocular disease, genital herpes, encephalitis and neonatal infection. Gingivostomatitis and pharyngitis are the most frequent clinical manifestations of orofacial HSV infections, usually involving the lip, tounge, gingiva, palate and face. First-episode infections lead to the formation of exulcerating vesicular lesions in the epithelia, and the clinical symptoms include pain, itching and fever. Recurrent herpes labialis is the most frequent clinical consequence of reactivation. Recurrent herpes labialis is associated with feaster healing and less frequent systemic signs than primary orofacial herpes. Although both viral types can invade the oral mucosa and lips, HSV-1 is the predominant causative agent of orofacial herpetic infections. (118, 152)

Genital herpes is a sexually transmitted disease primarily associated with HSV-2. HSV genital tract disease is characterized by local and systemic signs, including pain, itching, dysuria, vaginal and urethral discharge, inguinal lymphadenopathy, fever, headache, malaise, and myalgias. HSV replicating in external genitalia triggers the development of epithelial lesion and intensive inflammatory reaction. The lesions vary in their stages; vesicles, pustules, painful erythematous ulcers, crusting, or re-epithelialization can all be observed. Occasionally, endometritis, salpingitis and prostatitis may also arise from genital HSV infection. Genital herpes is frequently accompanied by local spread and systemic complications. The infection frequently extends to the buttock, groin and thigh area, and rarely the fingers and the eye can also be involved. HSV-2 may even cause

autonomic nervous system dysfunction, transverse myelitis, and meningitis with headache, stiff neck and photophobia. (152-154)

Herpetic eye involvement may manifest clinically as blepharitis, conjunctivitis, keratitis, iridocyclitis and acute retinal necrosis. Primary herpetic ocular surface disease can develop directly via 'front-door' route infection by droplet spread, or via a 'back-door' route, which involves the indirect spread of the HSV to the eye from a non-ocular site. Patients with herpetic conjunctivitis typically present with an acutely red eye, a watery discharge, conjunctival swelling, and in some cases photophobia and a foreign-body sensation. Occasionally, patients also exhibit subconjunctival hemorrhage and numerous lymphoid follicles on the tarsal conjunctiva. HSV-1 and HSV-2 may infect the cornea, leading to epithelial, stromal and endothelial keratitis, respectively. Epithelial keratitis can be characterized by the appearance of branching dendritiform, or enlarged geographic ulcers. Stromal keratitis and endothelitis can result in stromal scarring, thinning, neovascularization, severe iridocyclitis and an elevated intraocular pressure. (155, 156) Most cases of corneal ulceration will eventually resolve, though recurrent infections impair the corneal function and lead to a vision impairment that may even necessitate penetrating keratoplasty. Although HSV-1 continues to be the dominant type in ocular infections, HSV-2 also has the capability to invade the eye, and even mixed infections with both HSV-1 and HSV-2 can occur. The recent changes in behavioral patterns and sexual practices have led to a genital herpes outbreak, therefore a growing incidence in the ocular manifestations of HSV-2 infection may emerge in the future. (155-159)

Occasionally, HSV infection may appear in other clinical presentations. Reactivation of HSV infection or reinfection with another strain of HSV may cause encephalitis. Some visceral organs, such as the esophagus, lung and liver may also be infected with HSV, most commonly as part of multi-organ involvement in immunosuppressed patients. (152, 154, 160)

Neonatal herpes is acquired perinatally, congenitally or postnatally in 90%, 8% or 2% of the cases, respectively. Perinatal HSV infection usually results from contact with HSV-2-infected genital secretions during delivery. Congenital infection occurs in infants born to mothers who had primary HSV infection during pregnancy. Infants born to seronegative mothers may acquire infection through postnatal contact with symptomatic or asymptomatic HSV-1-infected family members or health care workers.

Neonatal herpes is severe, and has a tendency for dissemination to the central nervous system and visceral organs. (152, 161)

Several antiviral agents are available for the treatment of HSV infections. Aciclovir and its related compounds valaciclovir, penciclovir and famciclovir are the mainstay of therapy. Aciclovir (9-[(2-hydroxyethoxy)methyl]-9H-guanine) and penciclovir (9-[4-hydroxy-3-hydroxymethylbut-1-yl] guanine) are deoxyguanosine analogues. Valaciclovir is the l-valyl ester prodrug of aciclovir. Famciclovir is the diacetyl ester prodrug of 6-deoxy penciclovir. Following their cellular uptake, aciclovir and its related compounds are selectively phosphorylated to monophosphate forms by the thymidine kinase (TK) of HSV-1 and HSV-2, present only in infected cells. The drug monophosphates are then phosphorylated to triphosphate forms that act as competitive inhibitors of viral DNA polymerase. Moreover, aciclovir triphosphate is incorporated into the growing DNA chain of HSV, and causes chain termination, while penciclovir is not an obligate chain terminator. (118, 162) TK-deficient or TK-altered HSV strains may display resistance to these guanosine analogues. Thus, for the treatment of infections caused by viral isolates with altered TK cidofovir or foscarnet may be used. (162)

Cidofovir (S)-1-[3-hydroxy-2-(phosphomethoxy) propyl] cytosine dihydrate) is an acyclic phosphonate nucleotide analogue of deoxycytidine monophosphate, while foscarnet (trisodium phosphonoformate) is an inorganic pyrophosphate derivative. Cidofovir is phosphorylated to the active cidofovir diphosphate form by cellular enzymes, and thereby it is effective in infections caused by TK-altered HSV strains. Cidofovir diphosphate acts as competitive inhibitor of HSV DNA polymerase and chain terminator. Foscarnet does not undergo significant intracellular metabolism, instead it directly inhibits HSV DNA polymerase via blocking the pyrophosphate binding site on the enzyme. The selectivity of foscarnet lies in its greater efficiency against the viral DNA polymerase compared with the cellular enzymes. (118, 162, 163)

To date, no licensed vaccine is available for the prevention of HSV infections; replication-defective HSV-2, gD- or DNA-based vaccines are under clinical evaluation. (164-166)

AIMS

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

In an effort to gain more insight into the pathogenic mechanisms of RV infections, we set out to investigate viral multiplication, autophagy and apoptosis in RV-infected SIRC cell cultures. Our aims were as follows:

a.) To investigate the susceptibility of the SIRC corneal cell line to RV.

b.) To study the effect of RV on basal and rapamycin-induced autophagy.

c.) To measure the autophagic flux in RV-infected cells.

d.) To analyze the effect of RV on autophagosome formation.

e.) To study the connection between autophagy and apoptosis in cells infected with RV.

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

In an effort to gain more insight into the pathogenic mechanism of herpetic ocular surface disease, we set out to investigate viral multiplication, autophagy and apoptosis in cultures infected with HSV-1 or HSV-2. Our aims were as follows:

a.) To investigate the susceptibility of the SIRC corneal cell line to HSV-1 and HSV-2.

b.) To study the effects of HSV-1 and HSV-2 on basal autophagy.

c.) To measure the autophagic flux in cells infected with HSV-1 or HSV-2.

d.) To analyze the effects of HSV-1 on autophagosome formation.

e.) To study the connection between autophagy and apoptosis in cells infected with HSV-1 or HSV-2.

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 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum (Lonza, Verviers, Belgium).

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

Viruses

HSV: 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 units 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.

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.

Methods used to detect viral replication

RV: Virus plaque assay was performed on confluent monolayers of Vero cells inoculated with virus solution for 1 h at 35 °C and overlaid with 0.5% agarose (FMC, Rockland, ME, USA) 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 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA and Sigma-Aldrich, respectively) . After washing with PBS, the samples were reacted with fluorescein isothiocyanate (FITC)-conjugated or CF640R-labeled species-specific antibodies (Sigma-Aldrich) 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. (167) 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. (167)

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 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 (168) operated in the Image J software. (167)

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

For apoptosis ELISA, the cells were processed in a cell death detection ELISA kit (Roche Diagnostics GmbH, Penzberg, Germany). 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) (MBL, Woburn, MA, USA) 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 (Novagen, EMD Biosciences, Darmstadt, Germany), 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 (Bio-Rad, Hercules, CA, USA). 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 (Bio-Rad). Preblocked blots were reacted

with antibodies against actin (Sigma-Aldrich), HSV gD (Sigma-Aldrich), LC3B (Sigma-Aldrich) and Atg5 (Santa Cruz Biotechnology, Inc.) for 4 h in PBS containing 0.05% Tween 20, 1% dried non-fat milk (Difco Laboratories, Detroit, MI, USA) and 1% BSA (Sigma-Aldrich). Blots were then incubated for 2 h with peroxidase-conjugated species-specific antibodies (DakoCytomation, Carpinteria, CA, USA). Filters were developed by using a chemiluminescence detection system (Thermo Fisher Scientific Inc., Rockford, IL, USA). The autoradiographs were scanned with a GS-800 densitometer (Bio-Rad), 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 (Fig. 1a) in 6.2, 42.8 and 69.1 % of the cells, respectively. In good accord with previous findings (169), 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. (Fig. 1b; lanes 1-4) In contrast, RV-infected cells exhibited only moderate increases in LC3B-II levels at 3 and 5 dpi. (Fig. 1b; lanes 5-8)

The endogenous expression of the Atg12–Atg5 conjugate in the mock-infected cells was slightly downregulated during the 5-day culture period (Fig. 1b; lanes 1-4), while RV infection triggered a pronounced decrease in the level of the Atg12–Atg5 conjugate. (Fig. 1b; lanes 6-8) 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 (data not shown), 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 (Fig. 1c; lanes 4 and 2, respectively), 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.

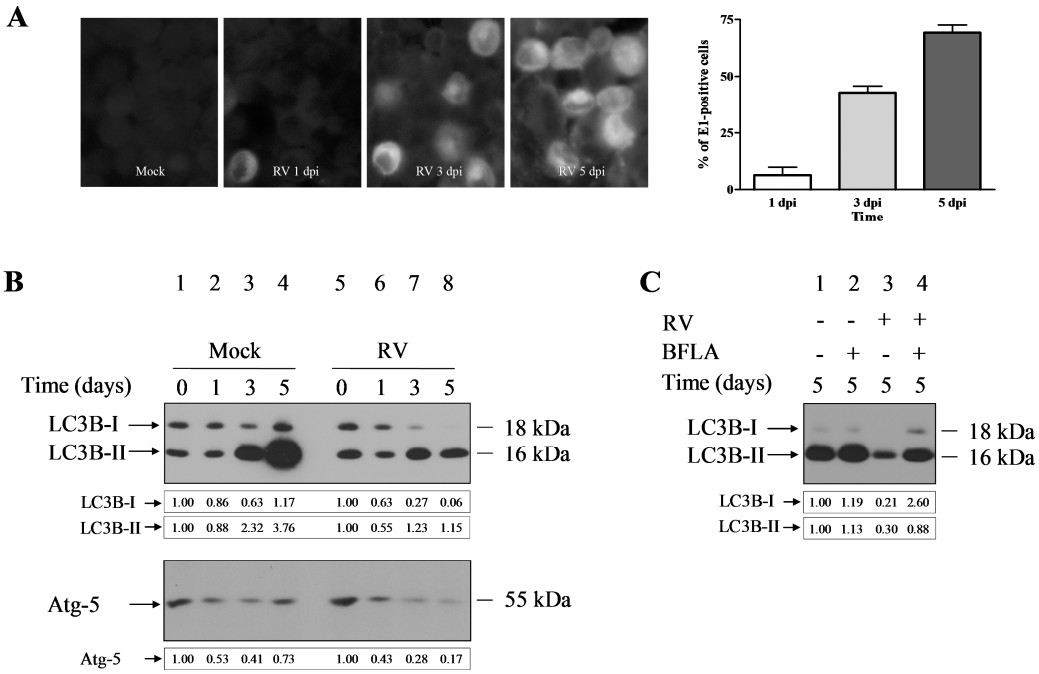

Fig. 1 RV infection decreases the level of autophagic proteins and impedes the autophagix flux. **a** Detection of RV E1 envelope glycoprotein by immunofluorescence assay. Scale bar 10 μm. Values on the graph denote mean ± SEM of three independent experiments. **b** Western blot analysis showing the kinetics of endogenous LC3B-II and Atg12-Atg5 conjugate expression. Band intensities were quantified by using the ImageQuant Software. The ratios of the protein levels measured on days 1, 3 and 5 to that of the corresponding 0-day time point controls were calculated, and expressed as fold change, shown below each lane. **c** Western blot analysis showing reduced autophagic flux in RV-infected SIRC cells. Mock- and RV-infected cells were either exposed to 100 nM bafilomycin A1 or left untreated. The total protein extracted was analyzed for LC3B expression by Western blot analysis. *BFLA* bafilomycin A1

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. As rapamycin exerted cytotoxic effects when it was present throughout the infection (data not shown), the cultures were infected first and treated with this autophagy inducer for a 24-h period just before the preparation of cell lysates. Both mock-infected and rapamycin-treated cells displayed patterns characterized by the combination of a diffuse, faint and punctate, bright LC3B staining. (Fig. 2a, c, respectively) 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. (Fig. 2a, c, 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. (Fig. 2b) Moreover, the intensity level of LC3B staining in RV-infected cells

treated with rapamycin was much lower than that of the drug control. (Fig. 2d, c, respectively) 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. (Fig. 2e, f) The average numbers of LC3B-positive vesicles per cell in the mock-infected and rapamycin-treated cultures were 19.76 and 21.89, respectively. (Fig. 2e) 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. (Fig. 2e) Thus, RV infection triggers a significant decrease in the accumulation of autophagosomes. (Fig. 2e) The average Feret diameters of LC3B-positive vesicles in the mock-infected and rapamycin-treated cultures were 657 and 680 nm, respectively. (Fig. 2f) 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. (Fig. 2f) Thus, RV infection elicits a significant decrease in the size of the autophagosomes. (Fig. 2f)

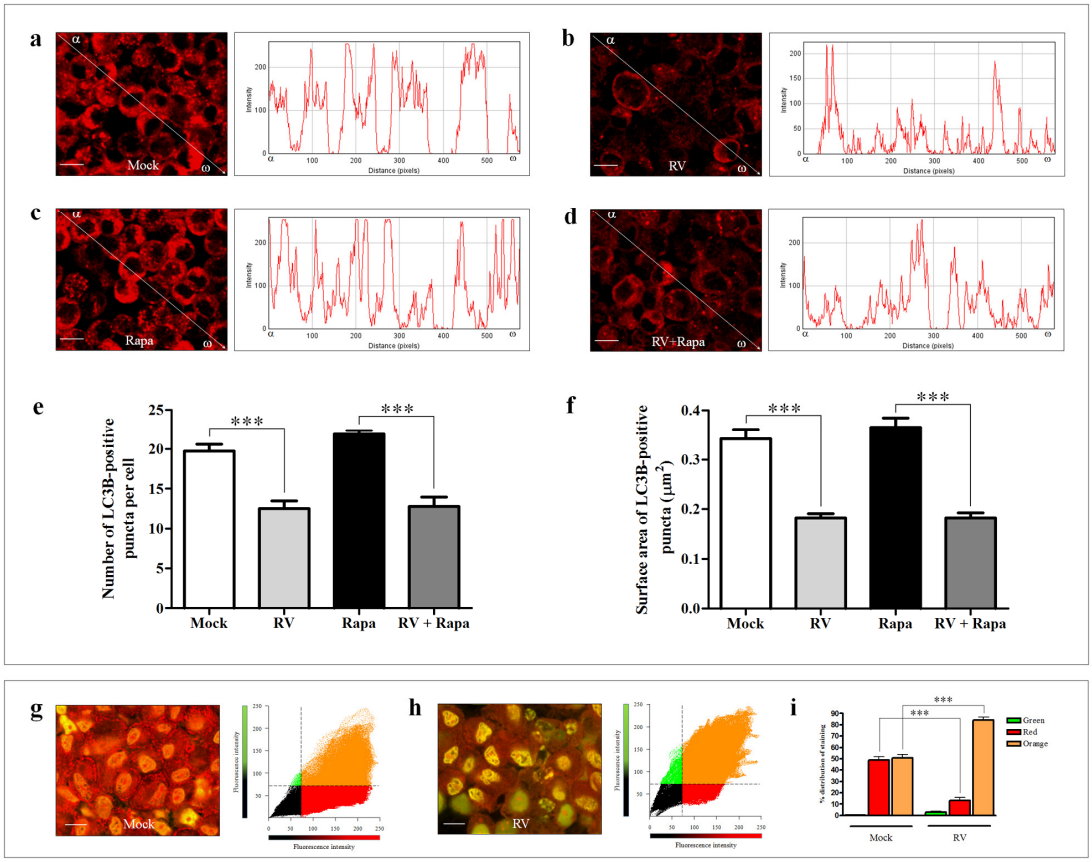

Fig. 2 RV infection retards autophagosome formation. SIRC cells were infected with the To336 strain of RV at an MOI of 5 for 5 days, and the intracellular localization of LC3B and cytoplasmic acidification were analyzed. **a-d** Immunofluorescence assay showing the fluorescence intensity of

LC3B-positive autophagosomes. The images were subjected to line scan fluorescence intensity analysis by using the Image J software. (167) The graphs depict the intensity values along the arrows drawn across the images. **a** Mock-infected culture; **b** RV-infected culture; **c** cells treated with rapamycin; **d** RV-infected culture treated with rapamycin. Scale bar 10 μm. **e** Immunofluorescence assay showing the average number of LC3B-positive autophagosomes. The LC3B-positive autophagosomes were automatically quantified by using the Image J software. (167) The values on the bar graph denote the mean ± SEM of the results of three independent experiments. P values were calculated by the Student's unpaired t test. *** $P < 0.001$. **f** Immunofluorescence assay showing the average size of LC3B-positive autophagosomes. The surface area of the LC3B-positive autophagosomes was determined by using the Image J software. (167) The values on the bar graph denote mean ± SEM of the results of three independent experiments. P values were calculated by the Student's unpaired t test. *** $P < 0.001$. **g-i** Reduced AVO formation in RV-infected cells stained with AO. Mock- and RV-infected cells were stained with AO, and images were obtained by confocal microscopy. AO accumulated by acidic compartments emits orange fluorescence, whereas this metachromatic dye bound to compartments with slightly acidic or neutral pH emits green fluorescence. Fluorescence intensities in green, red and overlapping spectral regions were determined and analyzed by using an „apoptosis correlator" plugin (168) operated in the Image J software. (167) Accordingly, the non-overlapping signals are green or red, while the overlapping pixels appear as orange in the correlation plots. Thresholds indicated by dashed lines were chosen empirically so as to separate visible fluorescence in both images obtained in green and red spectral regions from the dark pixels. The values on the bar graph denote mean ± SEM of the results of five independent experiments. P values were calculated by Student's unpaired t test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Rapa, rapamycin. Scale bar 10 μm.

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. (Fig. 2g) 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. (Fig. 2h) 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. (Fig. 2g-i) 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. With regard to its toxicity, rapamycin was added to RV-infected cultures for only 24 h just before the preparation of cell lysates. Rapamycin triggered an increase in

LC3B-II expression as compared with the mock-infected control. (Fig. 3a; lanes 4 and 1, respectively) This result, corroborating the data obtained by using immunofluorescence analysis (Fig. 2a), 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. (Fig. 3a; lanes 3 and 2, respectively) 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. (Fig. 3b) 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. (Fig. 3c) 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. (Fig. 3c) 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. (Fig. 3c) Thus, rapamycin-mediated induction of autophagy inhibits the apoptosis triggered by RV.

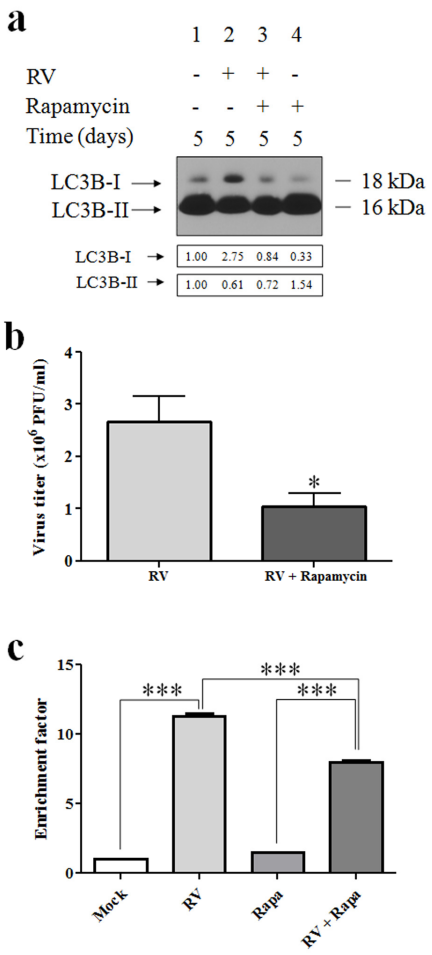

Fig. 3 Stimulation of autophagy inhibits viral replication and decreases apoptosis in RV-infected cells. **a** Western blot analysis showing the expression level of LC3B. Mock- and RV-infected cells were either exposed to 100 nM rapamycin or left untreated. Band intensities were quantified by using the ImageQuant Software, and the fold change is shown below each lane. **b** Plaque assay showing decreased RV plaque titers in response of rapamycin treatment. Values denote mean ± SEM of the results of three independent experiments. P values were calculated by the Student's unpaired t test. * $P < 0.05$. **c** ELISA showing decreased apoptosis of RV-infected cells in response of rapamycin treatment. Apoptosis was detected by measuring the specific mono- and oligonucleosomes in the cytoplasm by ELISA. The enrichment factor was calculated as described in the „Materials and methods" section. Values denote mean ± SEM of the results of three independent experiments. P values were calculated by the Student's unpaired t test. *** $P < 0.001$. Rapa, rapamycin.

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. (Fig. 4A) 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. (Fig. 4B)

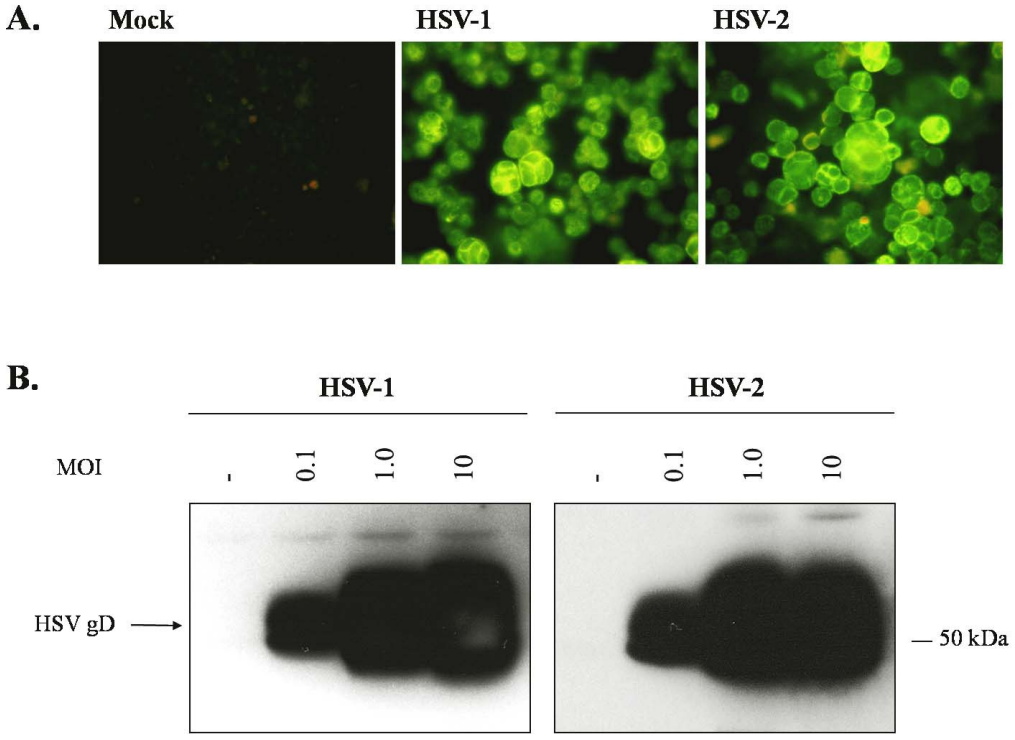

Fig.4. Replication of HSV-1 and HSV-2 in SIRC cells. (**A**) Detection of HSV gD by immunofluorescence assay. (**B**) Detection of HSV gD by Western blot assay.

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. (Table 1) The level of HSV-2 production varied between 6.8 x 10^4 and 3.7 x 10^7 PFU/ml. (Table 1) Together, these data demonstrate that the SIRC cell line is permissive for both HSV-1 and HSV-2 infection.

MOI	Titer of HSV-1 (PFU/ml)	Titer of HSV-2 (PFU/ml)
0.1	2.3 x 10^5	6.8 x 10^4
1	5.4 x 10^5	5.2 x 10^5
10	3.1 x 10^7	3.7 x 10^7

Table 1. Viral titers in HSV-1- and HSV-2-infected SIRC cells

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. (Fig. 5A) In contrast, there were numerous intracytoplasmic vacuoles in HSV-1-infected cells. (Fig. 5B-D) These vacuolar structures contained amorphous materials, organelles or virion particles. (Fig. 5B-D) The vacuoles were dispersed in the cytoplasm evenly. (Fig. 5B-D) 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. (Fig. 5B)

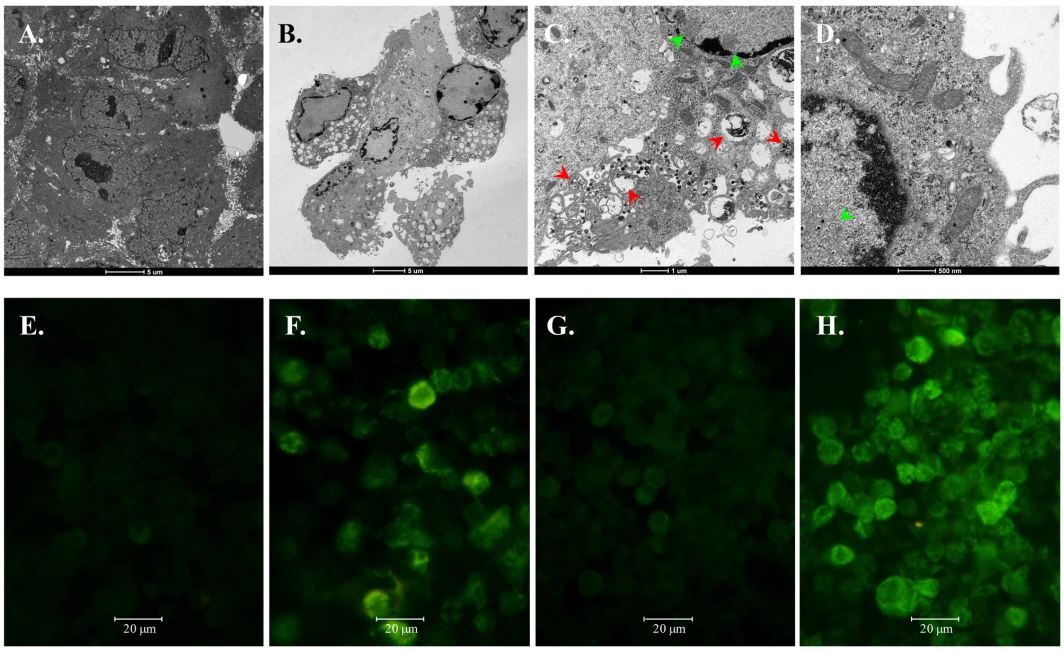

Fig. 5 HSV-1 infection increases the formation of autophagic vacuoles in SIRC cells. (**A-D**) Formation of autophagic vacuoles as assessed by TEM. (A) Mock-infected cells; (**B**, **C** and **D**) cells infected with HSV-1. Red and green arrows indicate autophagic vacuoles and viral particles, respectively. (**E** and **F**) Immunofluorescence assay for localization of Atg5. (**E**) Mock-infected cells; (**F**) cells infected with HSV-1. (**G** and **H**) Immunofluorescence assay for localization of LC3B. (**G**) Mock-infected cells; (**H**) cells infected with HSV-1.

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. (Fig. 5E and G, respectively) 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. (Fig. 5F and H, 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 was used as a pharmacological tool to inhibit lysosomal degradative activity and to prevent fusion of autophagosomes with lysosomes. As bafilomycin A1 decreased the production of infectious virions severely when it was present throughout the infection (data not shown), an experimental strategy that allows adsorption and uncoating to proceed undisturbed was applied. (Fig. 6A) (170) 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 bafilomycin A1 for an additional 12 h period, and analyzed for viral yields, apoptosis and LC3B expression. (Fig. 6A) 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. (Fig. 6B) This result is fully consistent with previous observations demonstrating that gD migrates as a doublet; the ~52 and ~56 kDa polypeptides correspond to the non-glycosylated and fully glycosylated forms of gD, respectively. (171-173) Bafilomycin A1 inhibited the accumulation of this envelope glycoprotein in HSV-1- or HSV-2-infected cells by 12.3% and 42.5%, respectively. (Fig. 6B) Plaque assay to quantify viral multiplication revealed that bafilomycin A1 retarded replication of HSV-1 and HSV-2 by 52.6% and 13.6%, respectively. (Fig. 6C) Thus, the strategy involving delayed addition of bafilomycin A1 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. (Fig. 6D, lane 6) 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. (Fig. 6D, lane 2 and 4, respectively) This result

demonstrates that HSV-1 and HSV-2 promote LC3B lipidation. Bafilomycin A1 highly elevated the level of LC3B-II (Fig. 6D, lane 1), 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 bafilomycin A1, HSV-1 and HSV-2 triggered slightly lower increase in the level of LC3B-II than that observed in the corresponding drug control. (Fig. 6D, lane 3, 5 and 1, respectively) Thus, both HSV-1 and HSV-2 decrease the autophagic flux to some extent.

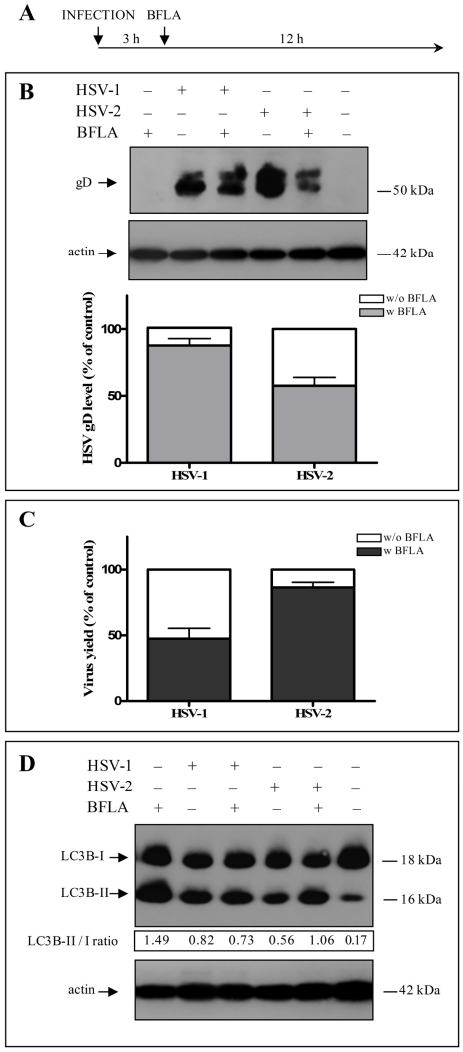

Fig. 6 HSV-1 and HSV-2 trigger LC3B lipidation, without increasing the autophagic flux. (**A**) Graphical scheme of the experiments designed to assess autophagic flux. (**B**) Detection of HSV gD and actin by Western blot assay. Relative densities of HSV gD and actin were measured by densitometric analysis of western blots. HSV gD was normalized to actin, percentage changes of gD levels in HSV-infected cells incubated with BFLA were compared to infected controls incubated in the absence of BFLA, and the results are presented in graph form below the image. (**C**) Detection of viral yield by plaque assay. (**D**) Detection of LC3B and actin by Western blot assay. Relative densities of LC3B-I, LC3B-II and actin were measured by densitometric analysis of western blots. LC3B-I and LC3B-II were normalized to actin, and the LC3B-II to LC3B-I ratio is shown below each lane.

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. (Fig. 7A) The nuclei of mock-infected cells were mostly round in shape. (Fig. 7A) 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. (Fig. 7A) 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. (Fig. 7A) 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. (Fig. 7A) Thus, HSV-1 and HSV-2 trigger the development of AVOs.

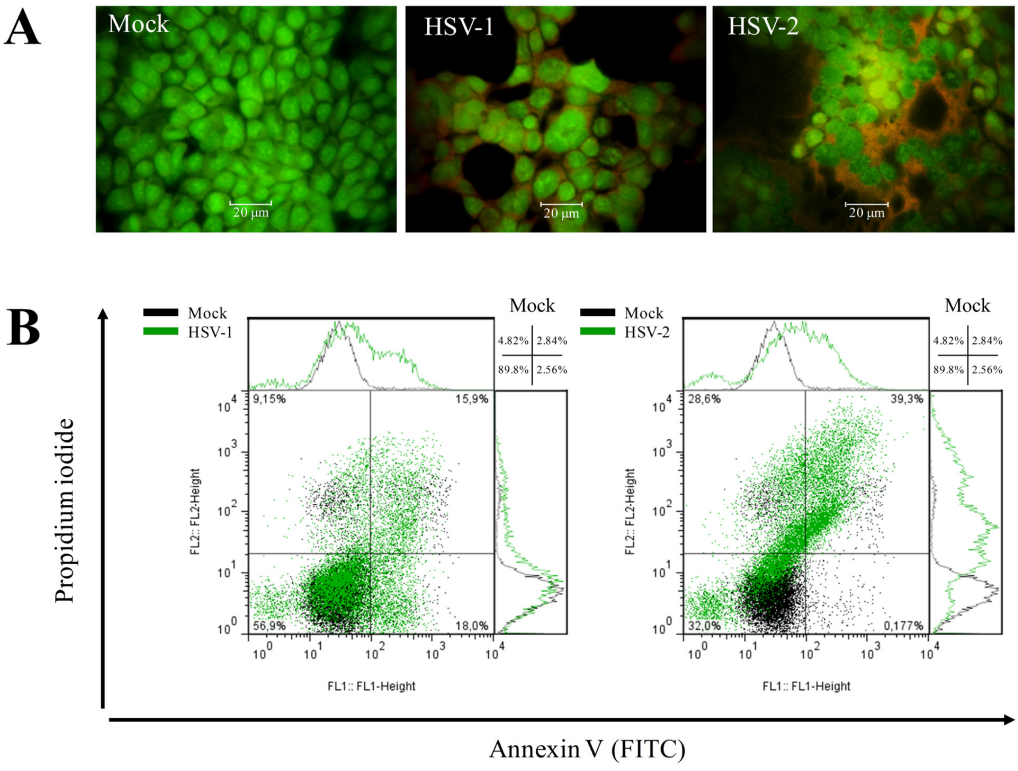

Fig. 7 HSV-1 and HSV-2 trigger cytoplasmic acidification and excessive cell death in SIRC cells. (**A**) Intracellular pH as assessed by acridine orange staining. (**B**) Cell death pattern as assessed by AN/PI double labelling. The fluorescence intensities of AN-FITC and PI were determined by flow cytometry. The two grpahs show superimposed dot plots of fluorescence intensities either of HSV-1 or HSV-2 and mock-infected cells.

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. (Fig. 7B) 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. (Fig. 7B) 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. (Fig. 7B) 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 bafilomycin A1 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 bafilomycin A1 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. (Fig. 8) EFs for the HSV-1- and HSV-2-infected cells incubated in the presence of bafilomycin A1 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. (Fig. 8) Thus, bafilomycin A1 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 bafilomycin A1-treated cells. (Fig. 8) Together, these results demonstrate that inhibition of autophagy facilitates apoptosis induced by HSV-1 and HSV-2.

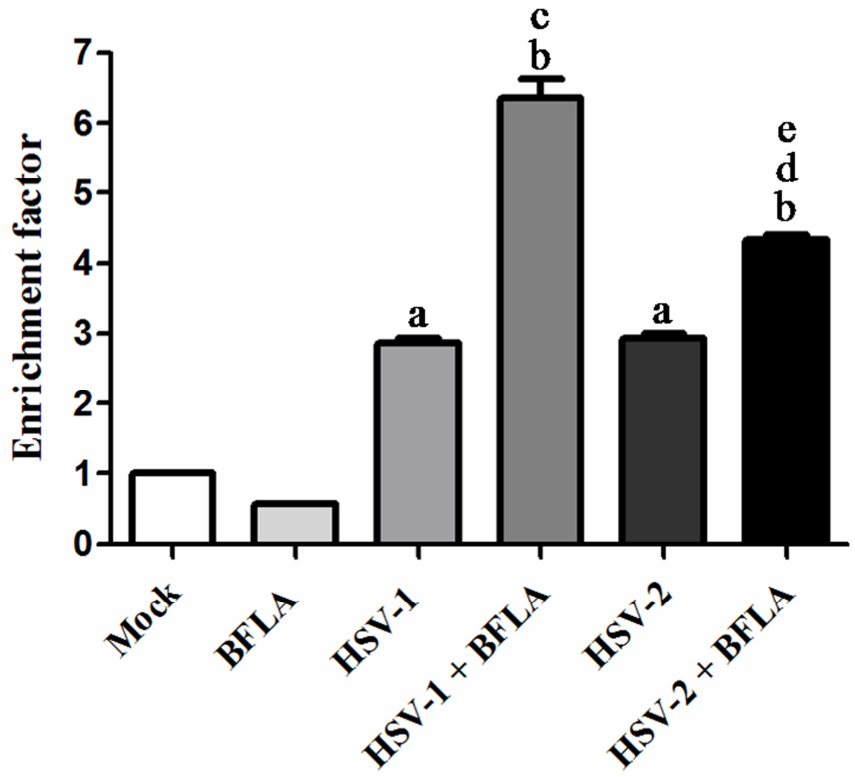

Fig. 8 Bafilomycin A1 decreases apoptosis in HSV-1- and HSV-2-infected cells. Apoptosis was detected by ELISA. The enrichment factor was calculated as described in the "Materials and methods". Data are mean (±SD) values from three independent experiments. P values were calculated by the ANOVA test with the Bonferroni post-test. [a] p< 0.001 vs Mock; [b]p<0.001 vs BFLA; [c]p<0.001 vs HSV-1; [d] p<0.001 vs HSV-2; [e]p<0.001 vs HSV-1+BFLA

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. (174) 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. (175-181) Further interesting studies have revealed that chikungunya virus, belonging in the *Alphavirus* genus in the family *Togaviridae*, elicits ER stress and oxidative damage, which in turn activate autophagy via the inositol-requiring enzyme 1α/X-boxbinding protein 1 (IRE1α/XBP1) pathway in conjunction with mTOR inhibition. (182, 183) Although the autophagy induced by chikungunya

virus facilitates viral replication, it delays apoptotic cell demise and mortality after infection. (183,184) Thus, autophagy has a protective role in the pathogenesis of acute chikungunya disease. (182-186) Another alphavirus, sindbis virus, has been shown to activate selective autophagy that involves p62/SQSTM1-Sma and Mad-related family ubiquitin regulatory factor 1 (SMURF1)-mediated targeting of capsid protein and assembled nucleocapsids for autophagic degradation. (187) Sindbis virus-induced selective autophagy has been demonstrated to be insufficient to restrain the robust viral multiplication, possibly in consequence of its overwhelmed clearance capacity. (188, 189) However, the autophagic removal of capsid protein aggregates and excess nucleocapsids significantly decreases Sindbis virus-induced cell death and dramatically improves the survival of the infected animals. (188) 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 (190) 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 (Fig. 1b), and this was accompanied by a decrease in the Atg12–Atg5 conjugate level at 3 and 5 dpi (Fig. 1b). 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 bafilomycin A1. In the presence of bafilomycin A1, the LC3B-II level of RV-infected cells was substantially lower than that seen in the drug control (Fig. 1c), 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 (Fig. 2a–f), 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 (Fig. 2g–i). 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. (190) Further studies have also revealed that defects in LC3 function lead to the failure of autophagosome closure. (191)

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). (192) 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. (192) 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. (193-196) 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 (Fig. 3b) 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 (Fig. 1a). (81, 99, 100) 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 (197), 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 (Fig. 3c). 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. (198)

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. (148, 199-201) 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. (148-150, 199, 202) Further interesting studies have revealed that varicella-zoster virus, lacking genes encoding anti-autophagic proteins, activates autophagy *in vitro* and in zoster vesicles. (203) A great body of experimental evidence also indicates that human cytomegalovirus (CMV) enhances some specific steps in the autophagic cascade, while it inhibits others. (201) 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. (147) 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. (201, 204, 205) 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. (206) 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 (Fig. 5B-D), as well as the recruitment of LC3B and Atg5 to autophagic vacuoles in the SIRC cell line (Fig. 5E and G). Our further experiments have also shown that both HSV-1 and HSV-2 trigger LC3B lipidation (Fig. 6D, lane 2 and 4), and increase cytoplasmic acidification (Fig. 7A), 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. (207-210) 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. (208, 211-215) 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. (208, 216) 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. (208, 217-219) In full accord with

these findings our data have corroborated that both HSV-1 and HSV-2 trigger apoptosis in corneal cells (Fig. 7B and Fig. 8).

Recent findings demonstrated that the apoptotic, autophagic and necrotic pathways are interconnected and subject to coordinated regulation. (220) Autophagy can operate as antagonist, facilitator or cooperator of apoptosis depending on the nature of stressor and cellular context. (220) 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. (221) 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 (Fig. 8) 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 (221), 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. (222) 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

- RV inhibits autophagy in SIRC cell line.
- RV blocks the autophagic flux.
- RV inhibits the development of autophagosomes.
- RV inhibits cytoplasmic acidification.
- Activation of autophagy inhibits RV-mediated induction of apoptosis.
- HSV-1 facilitates the accumulation of autophagosomes, as well as the recruitment of LC3B and Atg5 to autophagic vacoules in the SIRC cell line.
- HSV-1 and HSV-2 stimulate autophagy, without increasing the autophagic flux in the SIRC cell line.
- HSV-1 facilitates the accumulation of autophagosomes.
- HSV-1 and HSV-2 increase cytoplasmic acidification.
- Inhibition of autophagy enhances apoptosis triggered by HSV-1 and 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 bafilomycin A1 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.

ÖSSZEFOGLALÁS

A vírusok szaporodásuk során számos intracelluláris folyamat működését befolyásolják. Ezen változások különféle programozott sejthalál formák aktiválódását eredményezhetik. Vírusfertőzések során az apoptózis, az anoikis, a nekroptózis, a partanatosz, a piroptózis és az autofágia indukcióját írták le.

Az autofágia finoman szabályozott lebontó folyamat, mely eltávolítja és elemeire bontja a károsodott sejtalkotókat és fehérjéket. Intracelluláris mikroorganizmusok megsemmisítését is végzi, ez esetben a folyamatot xenofágiának nevezik. Az autofágia során az eltávolítandó sejtkomponensek, mikroorganizmusok az autofagoszómába kerülnek, ami a lizoszómával való fúzióját követően autolizoszómává alakul. A vezikulatartalom a lizoszómális enzimek hatására lebomlik. Az autofagoszóma kialakulásában fontos szerepet játszik az Atg5, Atg12 és Atg16L proteinek által alkotott, 800 kDa molekulatömegű szuperkomplex, valamint az LC3B ubikvitinszerű fehérje. Az autofagoszóma kialakulása során először az Atg4 cisztein proteáz a pro-LC3B-t LC3B-I-re hasítja. Az LC3B-I-hez az Atg5/Atg12/Atg16L komplex foszfatidil-etanolamint kapcsol, és így jön létre az LC3B-II, ami nélkülözhetetlen az autofagoszóma membrán felépüléséhez.

A vírusok autofágiára gyakorolt hatása még nem kellően tisztázott. Vizsgálataink során ezért a rubeolavírus és a herpes simplex vírusok autofágiás folyamatokra gyakorolt hatásait tanulmányoztuk *in vitro* SIRC corneális sejtvonalon.

A rubeolavírus autofágiára gyakorolt hatása

Munkánk kezdeti szakaszában indirekt immunfluoreszencia és plakk titrálás segítségével megállapítottuk, hogy a SIRC sejtvonal fogékony a rubeolavírus fertőzésre.

Ezt követően western blot analízissel tanulmányoztuk az LC3B-I és -II proteinek valamint az Atg5–Atg12 komplex szintjét. A fertőzetlen kultúrákban a 0. napon az LC3B mindkét formája kimutatható volt, és az LC3B-II szintje jelentős emelkedést mutatott a kontroll 1., 3., és 5. napos mintáiban. Ezen adat bizonyította a bazális autofágia aktiválódását a kontroll sejtekben. Ezzel szemben, a vírussal fertőzött kultúrákban az LC3B-II szintjének csak kismértékű emelkedését tudtuk detektálni a fertőzést követő 3. és 5. napon. A kontroll minták endogén Atg5–Atg12 expressziója kismértékű csökkenést mutatott, míg a vírusfertőzés hatására az Atg5–Atg12 komplex szintje nagymértékben csökkent a vírusfertőzéssel párhuzamosan. A rubeolavírus tehát

nagymértékben csökkenti az Atg5–Atg12 fehérje szintjét és gátolja az LC3B-I lipidációját.

Az autofágiás-fluxus vizsgálatára irodalmi adatoknak megfelelően bafilomycin A1 kezelést alkalmaztunk. Megállapítottuk, hogy a vírussal fertőzött és bafilomycin A1-gyel kezelt mintákban a lipidált LC3B szintje alacsonyabb volt, mint a bafilomycin A1 kontrollé. A rubeolavírus tehát gátolja az autofágiás fluxust.

Az LC3B intracelluláris lokalizációját indirekt immunfluoreszcenciás vizsgálat segítségével tanulmányoztuk bazális és rapamycin-indukálta autofágia során. Az intracelluláris LC3B eloszlás, LC3B-I és LC3B-II esetében különbözik. Az LC3B-I a citoplazmában szabadon található meg, immunfestés során diffúz citoplazmafestődést láthatunk, míg lipidációt követően az LC3B-II beépül az autofagoszómák membránjába. Eredményeink szerint mind a kontroll, mind pedig a rapamycinnel kezelt sejtekben intenzív, pöttyözött LC3B festődés volt kimutatható az 5 napos kultúrákban; a számos nagyméretű autofagoszóma jelenlétének köszönhetően. Ezzel szemben a vírussal fertőzött mintákban, illetve a vírussal fertőzött és rapamycinnel kezelt kultúrákban lényegesen kevesebb autofagoszóma volt detektálható. A festődés intenzitását line scan analízissel kvantitáltuk. Mérési adataink bizonyították, hogy a kontroll és rapamycinnel kezelt mintákban számos erőteljes csúcs található, míg a vírusfertőzött illetve vírusfertőzött és rapamycin-kezelt mintákban sokkal kevesebb és kisebb intenzitású csúcs van jelen. A rubeolavírus tehát gátolja az LC3B lipidációját, és ezáltal az autofagoszómákba történő transzlokációját. ImageJ software segítségével meghatároztuk a vakuolumok számát és méretét. Az átlagos vakuolumszám a kontroll sejtek esetében 19,76, a rapamycinnel kezelt kultúrákban pedig 21,89 volt. A vírussal kezelt sejtekben az átlagos autofagoszómaszám 12,49, míg a vírussal-fertőzött és rapamycinnel kezelt kultúrákban 12,81 volt. A rubeolavírus tehát csökkenti az autofagoszómák számát. Az átlagos vakuolum átmérő a kontroll sejtek esetében 657 nm, a rapamycinnel kezelt kultúrákban 680 nm volt. A vírussal kezelt sejtekben az autofagoszómák átlagos átmérője 481, míg a vírussal-fertőzött és rapamycinnel kezelt kultúrákban 480 nm volt. Érdekes módon a rubeolavírus az autophagosomák átmérőjét is csökkenti.

Az autofagoszómák lizoszómákkal való fúziójának köszönhetően autofágia során a citoplazma pH-ja savi irányba tolódik. A citoplazmatikus acidifikációt acridin orange festés segítségével vizsgáltuk. Az acridine orange savi pH-nál piros, míg neutrális pH-nál zöld színű. A kontroll sejtek citoplazmájában intenzív, punktált piros festődés volt

látható, míg a vírusfertőzött sejtekben a festődés intenzitása és az acidotikus vezikuláris organellumok száma is csökkent. A kontroll kultúrákban, a különböző spektrális tartományokban mért festődési intenzitások megoszlása a piros szín esetében 49%, a narancs szín esetében 50.6%, a zöld szín esetében pedig 0,4% volt. A vírusfertőzött sejtekben, a festődési intenzitások megoszlása a piros szín esetében 13%, a narancs szín esetében 84%, a zöld szín esetében pedig 3% volt. A rubeolavírus tehát jelentősen csökkenti az acidotikus vezikuláris organellumok számát a fertőzött sejtekben.

Végül tanulmányoztuk azt is, hogy az autofágia milyen hatást gyakorol a vírus fertőzés folyamatára. Rapamycin alkalmazásával indukáltuk az autofágia mTOR útvonalát, és mértük az infektív virion számot, valamint az apoptosis mértékét. Plakk titrálással megállapítottuk, hogy az autofágia-indukció szignifikánsan csökkentette az infektív virionszámot. Apoptózis ELISA segítségével megállapítottuk, hogy az autofágia-indukció gátolja a rubeolavírus által indukált apoptózist. A rubeolavírus tehát két celluláris antivirális mechanizmust képes blokkolni az autofágia gátlása révén.

Mivel az autofágia fontos szerepet játszik az embriogenezis folyamatában, feltételezzük, hogy a rubeolavírus autofágiát gátló hatása hozzájárulhat a kongenitális rubeola szindróma során kialakuló súlyos magzati ártalomhoz.

A herpes simplex vírusok autofágiára gyakorolt hatása

Indirekt immunfluoreszencia segítségével megállapítottuk, hogy a SIRC sejtvonal fogékony mind a herpes simplex vírus-1 mind pedig a herpes simplex vírus-2 fertőzésre.

Transzmissziós elektronmikroszkópia segítségével tanulmányoztuk a herpes simplex vírus-1 fertőzés hatására létrejövő intranukleáris és intracitoplazmatikus replikációs kompartmentek, valamint a citoplazmában kialakuló auto- és xenofágiás vakuolumok jelenlétét. Ezen vizsgálatok egyértelműen bizonyították a virionok, valamint számos auto-, illetve xenofagoszóma jelenlétét a fertőzött sejtekben.

Az Atg5 és az LC3B intracelluláris lokalizációját indirekt immunfluoreszcenciás vizsgálat segítségével tanulmányoztuk bazális autofágia során. Kísérleteink a kontroll sejtekben halvány, diffúz citoplazmatikus festődést mutattak ki, ami a bazális autofágia alacsony aktivitására utal 24 órás sejtkultúrákban. Ezzel szemben, a vírussal fertőzött mintákban intenzív, pöttyözött Atg5 és LC3B festődés volt kimutatható; a

számos nagyméretű autofagoszóma jelenlétének köszönhetően. Ezen adatok arra utalnak, hogy a herpes simplex vírus-1 befolyásolja az Atg5 és az LC3B autofagoszómális marker proteinek intracelluláris lokalizációját.

Western blot analízissel tanulmányoztuk az LC3B-I és -II proteinek szintjét. A fertőzetlen kultúrákban az LC3B mindkét formája kimutatható volt a tenyésztés 15. órájában, és az LC3B-II alacsony szintje az autofágia kis mértékű alap aktivitására utalt. Az LC3B-II szintje jelentősen növekedett mind herpes simplex vírus-1, mind herpes simplex vírus-2 hatására. A fertőzés tehát fokozza az LC3B lipidációját.

Az autofágiás-fluxus vizsgálatára irodalmi adatoknak megfelelően bafilomycin A1 kezelést alkalmaztunk. Megállapítottuk, hogy a vírussal fertőzött és bafilomycin A1-gyel kezelt mintákban a lipidált LC3B szintje kissé alacsonyabb volt, mint a bafilomycin A1 kontrollé. A herpes simplex vírus-1 és a herpes simplex vírus-2 tehát valamelyest fékezi az autofágiás fluxust.

A citoplazmatikus acidifikáció mértékét a herpes simplex vírus fertőzés során is acridine orange festés segítségével vizsgáltuk. Megállapítottuk, hogy mind a herpes simplex vírus-1 mind pedig a herpes simplex vírus-2 fertőzés hatására fokozódik a citoplazmatikus acidifikáció mértéke.

Propidium jodid / annexin V kettős festéssel tanulmányoztuk a herpes simplex vírusok sejtkárosító hatását. Megállapítottuk, hogy az apoptotikus mechanizmusok fontos szerepet játszanak mind a herpes simplex vírus-1 mind a herpes simplex vírus-2 citopátiás hatásában.

Végül tanulmányoztuk az autofágia és az apoptózis közötti kölcsönhatást is. Bafilomycin A1-gyel gátoltuk az autofágiát, majd apoptózis ELISA-val mértük a sejtek herpes simplex vírus fertőzésre adott apoptotikus válaszát. Eredményeink szerint az autofágia gátlása jelentősen fokozta a mind a herpes simplex vírus-1 mind pedig a herpes simplex vírus-2 által indukált apoptózis mértékét.

Eredményeink arra engednek következtetni, hogy mind a herpes simplex vírus-1 mind pedig a herpes simplex vírus-2 autofágiát indukál a SIRC corneális sejtvonalon. Ezáltal a herpes simplex vírus fertőzések kiváltják az egyik legjelentősebb celluláris védekező mechanizmus, az autofágia fokozódását. Ezek az adatok hozzájárulhatnak a herpes simplex vírusok által okozott szemészeti és egyéb kórképek patogenezisének jobb megértéséhez is.

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Appendix