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Modulation of the Autophagy Pathway by Human Tumor Viruses

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Abstract

Autophagy is a highly conserved and regulated process in eukaryotic cells by which components of the cytoplasm, such as damaged organelles and foreign pathogens, become enveloped into double-membrane autophagosome vesicles that fuse with the lysosome for degradation. Viruses are adept at subverting host cellular pathways for their replication and survival. The human tumor viruses, Epstein-Barr virus (EBV), Kaposi's Sarcoma-Associated Herpesvirus (KSHV), Hepatitis B virus (HBV), and Hepatitis C virus (HCV), have evolved novel ways of modulating autophagy during productive and latent stages of the virus life cycle. This review will discuss how the autophagy pathway becomes activated upon viral infection and the role of viral proteins in regulating the autophagy pathway. Specifically, we will examine how virus-encoded homologs of autophagy proteins evade autophagy-mediated degradation by blocking the induction, elongation, or maturation steps in the autophagy pathway. We will also discuss how certain viruses enhance autophagy induction or usurp autophagic machinery for their own replication. A comprehensive understanding of the autophagic response to tumor viruses may enable the discovery of novel antiviral and/or anticancer drug therapies.

Keywords

autophagy; human tumor viruses; oncogene-induced senescence (OIS); unfolded protein response (UPR)

1. Introduction

Autophagy ("to eat itself") is an evolutionally conserved and highly regulated homeostatic process that involves the envelopment of cytoplasmic components into double-membrane autophagosome vesicles that fuse with the lysosome for degradation during nutrient starvation in a non-specific manner. This intracellular catabolic degradation system is tightly controlled by autophagy-related genes (*Atg*), which can initiate or suppress steps in the autophagy pathway in order to maintain cellular homeostasis. The serine/threonine kinase mammalian target of rapamycin (mTOR) is an important regulator of autophagy. Under normal conditions, mTOR represses autophagy induction by phosphorylating Unc-like

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Conflict of Interest

The authors declare that there are no conflicts of interest. Dr. Jung is not financially affiliated with Grifols Biologicals Inc.

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kinase 1 and 2 (ULK1/2). Nutrient starvation conditions or treatment with the mTOR inhibitor rapamycin impedes mTOR kinase activity, leading to autophagy initiation and nucleation of a phagophore membrane. During the initiation step of autophagy, Beclin-1 forms a complex with Vps34, a class III phosphoinositide 3-kinase (C3 PI 3-kinase), which contributes to autophagosome nucleation and assembly [1]. During the elongation step of autophagy, light chain 3 (LC3-I) is proteolytically processed and conjugated with a lipidated phosphatidylethanolamine (PE) via an ubiquitin-like conjugation system. Lipidated LC3-II can serve as a marker for autophagosome formation since LC3-II is embedded within the lumen of the autophagosome [2]. Autophagosomes subsequently undergo a maturation step by fusion with endosomes or lysosomes. The Beclin-1/Vps34/UVRA3 complex positively contributes to autophagosome maturation and endocytic trafficking [3,4]. Rab7 GTPase activity has also been shown to stimulate fusion of autophagosomes with late endosomes/lysosomes. The acidic environment in autolysosomes ultimately degrades the cargo by lysosomal hydrolysis.

Autophagy is an essential part of both the innate and adaptive immune systems. Xenophagy, a form of selective autophagy, specifically recognizes intracellular microbes and physically targets these pathogens to the autophagy pathway for degradation [5]. As a result, some pathogens have evolved multiple strategies to suppress autophagy, whereas others utilize autophagosome membranes and the autophagic machinery to enhance their replication. The gamma-herpesviruses Epstein-Barr virus (EBV) and Kaposi's Sarcoma-Associated Herpesvirus (KSHV) are oncogenic viruses that encode viral homologs of cellular autophagy proteins, which negatively regulate autophagy during latency, a stage in the life cycle where no progeny viruses are produced. In contrast, the tumor viruses Hepatitis B virus (HBV) and Hepatitis C virus (HCV) utilize components of the autophagy pathway to promote productive lytic replication. In this review we will focus on the function of autophagy as an intracellular defense mechanism and examine how human tumor viruses subvert multiple steps in the autophagy pathway to facilitate viral propagation and evade immune detection.

2. Activation of autophagy as a host response to viral infection

2.1 Induction of Autophagy by Cellular Stress Responses

Environmental stress responses, such as the accumulation of protein aggregates, damaged organelles, or intracellular bacteria and viruses, can induce selective autophagy. During productive viral replication, robust viral protein synthesis contributes to ER stress and activation of the unfolded protein response (UPR) pathway. UPR is characterized by the induction of the ER stress sensors ATF6, IRE1, and PERK as an attempt by stressed cells to regain homeostasis [6]. ER stress and UPR upregulation ultimately activate the autophagy pathway and autophagosome formation [7]. Both DNA and RNA viruses can indirectly trigger autophagy via UPR activation (Figure 1B). The DNA virus EBV encodes the oncogene latent membrane protein 1 (LMP1), which is required for proliferation in infected B cells. The six-transmembrane spanning domains (6TM) of LMP1 activate PERK, leading to UPR-mediated autophagy induction [8,9]. LMP1-induced autophagic degradation may serve as a mechanism to limit LMP1 accumulation in EBV-infected B cells. Expression of the small surface protein (HBs) of HBV can also induce the UPR pathway, leading to autophagy induction [10]. The positive-stranded RNA virus HCV induces the ER stress and UPR pathway, followed by autophagosome formation and accumulation [11]. HCV stimulates an incomplete autophagic response by inducing UPR-dependent autophagosome accumulation and suppression of autophagosome fusion with lysosomes [11]. Inhibition of ER stress by siRNA knockdown of ATF6, IRE1, or PERK suppressed LC3 lipidation, a marker of autophagosome formation, during HCV infection. The UPR-autophagy pathway positively contributes to HCV replication since siRNA knockdown of Atg5 or CHOP in

human hepatoma Huh7 cells inhibited early-stage synthesis of incoming HCV RNA [12]. Thus, HCV might usurp the UPR-autophagy pathway to use it as a mechanism of enhancing HCV RNA replication while inhibiting autolysosome maturation for protection from xenophagic degradation.

2.2 Oncogene-induced senescence (OIS) and autophagy

Acute oncogenic stress can also lead to activation of the autophagy pathway by a mechanism known as oncogene-induced senescence (OIS). Oncogene activation contributes to aberrant cellular proliferation, resulting in genotoxic stress and activation of DNA-damage responses (DDR). Chronic oncogene expression and DDR activation can trigger oncogene-induced senescence (OIS), a mechanism by which cells undergo permanent cell cycle arrest [13]. Autophagy-mediated enforcement of OIS via increased autophagic flux contributes to tumor suppression. KSHV establishes a life-long, persistent infection and only a subset of genes are expressed during latency. Latent KSHV infected cells display oncogenic stress and activation of DDR, yet remain refractory to senescence. Expression of the latent gene product viral cyclin (v-cyclin), a homolog of cellular D-type cyclins, deregulates cellular proliferation and triggers DDRs. Continued expression of oncogenic v-cyclin contributes to autophagy upregulation and the cells ultimately undergo cellular senescence if left unchecked [14]. Co-expression of v-cyclin with the KSHV latent gene product viral FLICE inhibitory protein (vFLIP) prevented OIS and v-cyclin-induced senescence [15,16]. The ATG3-binding domain of vFLIP was required for blockage of v-cyclin induced autophagy and senescence in cells latently infected with KSHV. The synergistic relationship between v-cyclin and vFLIP demonstrate an important role in blocking autophagy to prevent cellular senescence and facilitate proliferation of KSHV-infected cells (Figure 1C).

2.3 Autophagy and adaptive immunity during MHC antigen presentation

The innate immune system is a non-specific mechanism that generally detects foreign antigens while the adaptive immune system recognizes and initiates an antigen-specific immune response. Adaptive immunity retains specific pathogen memory via antigen presentation in order to mount an immune response during subsequent pathogen challenge. T lymphocytes become activated upon recognition of foreign antigens presented to them on major histocompatibility complex (MHC) molecules [17]. During MHC class II antigen presentation, exogenous peptides are processed by hydrolase degradation in lysosomal compartments and are presented on the cell surface of antigen-presenting cells to activate a CD4⁺ T cell response [18]. MHC class II-positive dendritic cells, B cells, and epithelial cells undergo steady-state autophagy to form autophagosomes that fuse with multivesicular MHC class II-loading compartments [19]. EBV is a persistent and life-long pathogen that maintains a latent state in memory B-cells. EBV can cause Burkitt's lymphoma, Hodgkin's lymphomas, B-cell post-transplant lymphoproliferative disorder, nasopharyngeal carcinoma, and gastric carcinoma [20]. The Epstein-Barr virus nuclear antigen protein (EBNA1) is the dominant CD4⁺ T cell antigen detected during latent EBV infection [21]. Treatment of EBNA1-specific CD4⁺ T cells with the autophagy inhibitor 3-Methyladenine or siRNA-knockdown of Atg12 led to down-regulation of endogenous MHC class II processing of EBNA1 [22]. In addition, EBNA1 accumulated in autophagosomes upon treatment with the lysosomal acidification inhibitors chloroquine or ammonium chloride [22]. EBNA1 subcellular localization within the nucleus limits autophagy-mediated endogenous MHC class II processing. Cytoplasmic targeting of EBNA1 by a nuclear localization signal mutant resulted in increased epitope presentation due to EBNA1 becoming more accessible to the autophagy and MHC class II pathways [23]. These studies confirm the importance of autophagy in antigen processing for MHC II presentation and how EBV limits its detection during latency, potentially contributing to EBV-positive tumorigenesis (Figure 1A).

3. Virus-mediated inhibition of the autophagy pathway

3.1 Inhibition of vesicle nucleation

Viruses have evolved various mechanisms to escape or inhibit multiple steps in the autophagy pathway (Figure 1A). Beclin-1 can function as either an activator or repressor of vesicle nucleation, depending on its protein interacting partners [3]. The Beclin-1/Bcl-2 complex represses vesicle nucleation by blocking C3-PI3-kinase activity. Most members of the gamma-herpesvirus family encode viral homologs of cellular Bcl-2 that are expressed during productive lytic infection. Although EBV encodes two ortholog proteins, BHRF1 and BALF-1, of cellular Bcl-2, the autophagy inhibiting function of these homologs remains unclear and has not been demonstrated. KSHV, the etiological agent of Kaposi's Sarcoma (KS), Primary Effusion Lymphoma (PEL), and Multicentric Castleman's disease (MCD) encodes orf16. Viral Bcl-2 homologs inhibit the nucleation step of autophagy by directly binding to Beclin-1 [24,25]. Based on structural and biochemical analysis of the murine gamma-herpesvirus 68 (MHV-68) viral Bcl-2 homolog, M11, lacks a phosphorylation site-containing regulatory loop, which is present in cellular Bcl-2, thereby resulting in stronger Beclin-1 binding affinity and efficient inhibition of autophagosome formation [26,27].

3.2 Inhibition of vesicle elongation and autophagosome formation

KSHV encodes a viral homolog of the cellular FLICE-like inhibitor protein (cFLIP), an inhibitor of death receptor-mediated apoptosis [28]. Both cellular FLIP and KSHV vFLIP directly interact with the E2-like Atg3 protein, a component of the LC3/Atg8 conjugation system. KSHV vFLIP functions as an inhibitor of autophagosome elongation during rapamycin- and starvation-induced autophagy by preventing Atg3 from binding and processing LC3 [29]. Small molecule peptides of the ten amino acid DED1 alpha2 helix and the twelve amino acid DED2 alpha4 helix of KSHV vFLIP were individually sufficient for Atg3 binding. *In vitro* and *in vivo* treatment with the vFLIP peptides resulted in autophagy-associated cell death in tumor cells by targeting cFLIP and increasing the interaction of Atg3 with LC3 [29]. Thus, acquisition of cellular Bcl-2 and FLIP homologs may be a strategy used by gamma-herpesviruses to subvert the autophagy pathway in order to enhance cell survival and maintain a persistent and life-long infection.

4. Autophagy as a mechanism of promoting virus replication

4.1 Autophagy-dependent membrane accumulation

In contrast to gamma herpesvirus-mediated suppression of the autophagy pathway to protect its lifecycle, HCV and HBV often induce autophagy and utilize the autophagic machinery to facilitate their replications (Figure 1A). HCV, a member of the flaviviridae family, infects more than 180 million people worldwide and is a causative agent of primary hepatocellular carcinoma [30]. Many positive-stranded RNA viruses utilize and induce the formation of double membrane vesicles (DMVs) to support viral replication. Indeed, accumulation of autophagic vacuoles has been observed in HCV-infected hepatocytes. DMVs derived from ER subdomains, which can be produced through autophagy and Atg5, have recently been identified as a source of membrane support for incoming HCV translation [31,33]. These DMVs contain HCV proteins, viral RNA, and LC3-II, suggesting that HCV activates the autophagy pathway for formation of autophagic vesicles as a membranous support for translation of incoming HCV RNA and enhancement of viral RNA replication. HCV induces autophagosome accumulation in an UPR-dependent, but C3-PI3K-independent manner [11]. Using a HCV subgenomic replicon cell line, HCV RNA replication was found to occur on autophagosome membranes. Inhibition of autophagosome formation by siRNA silencing of the autophagy-related genes LC3, Atg7, Atg4B, Atg12, or Beclin-1 suppressed HCV RNA replication in Huh 7.5 cells [31–33] and knockdown of the Beclin-1 or Atg7

inhibited the production of infectious virus particles in HCV-infected hepatocytes [34]. Incoming HCV RNA translation, but not the maintenance of progeny RNA replication, potentially requires the autophagy machinery. The HCV non-structural protein NS4B was recently shown to be sufficient for autophagy induction [35]. Co-immunoprecipitation studies confirmed that NS4B formed a complex with Rab5 and Vps34 [35]. Inhibition of Rab5 function by shRNA knockdown or Vps34 function by 3-Methyladenine treatment or shRNA knockdown impaired NS4B-induced autophagosome induction. Yeast two-hybrid and co-immunoprecipitation studies also identified an interaction between ATG5 and the non-structural protein NS5B [36]. The interaction of NS5B with Atg5 stimulated the conjugation step of LC3-I to PE. These studies suggest a potential role for NS4B and NS5B in the recruitment of Rab5 and Vps34 as a mechanism for HCV-induced autophagosome formation.

4.2 Autophagy and viral DNA replication

Autophagy can also enhance viral DNA replication. HBV is a small DNA virus with a partially double-stranded and circular DNA genome. Similar to HCV, HBV can also cause hepatocellular carcinoma. In contrast to HCV, HBV increases autophagic flux by inducing autophagy in a C3-PI3K-dependent manner. The HBV X (HBx) protein is necessary and sufficient to induce autophagy by binding to C3-PI3K, while concurrently minimizing autolysosomal protein degradation for enhancement of viral DNA replication *in vitro* [37]. The role of autophagy during *in vivo* HBV infection was examined in HBV transgenic mice with a liver specific knockout of Atg5 [38]. Ablation of autophagy led to minimal detection of HBV DNA replicative intermediates, confirming that autophagy is required for efficient HBV DNA replication *in vivo*. These observations suggest that some viruses hijack autophagy proteins to initiate or enhance viral replication.

5. Implications of autophagy inhibitors or inducers in cancer therapies

5.1 Autophagy-induction as a potential cancer therapy

Dysregulation of the autophagy pathway has been implicated in many human cancers. mTOR, a serine/threonine kinase that negatively regulates autophagy upstream of ULK1/2, is constitutively activated in Kaposi's sarcoma (KS) and KSHV-associated primary effusion lymphoma (PEL) [39]. Recent studies have examined the role of autophagy inducing drugs, such as the immunosuppressive mTOR inhibitor sirolimus (rapamycin), as a potential anti-tumor therapy. Sirolimus upregulates autophagy by binding to the FK506-binding protein (FKBP12) and this complex subsequently binds to mTOR, thereby inhibiting mTOR kinase activity. Using a PEL cell line and murine xenograft PEL model, sirolimus treatment was efficacious in inhibition of the cellular cytokines IL-6, IL-10, and interferon-gamma, thereby suppressing PEL cell growth *in vitro* and delayed PEL tumor progression *in vivo* [40]. Furthermore, Kaposi's sarcoma is a common complication following organ transplantation and immunosuppressive treatment. The clinical effect of sirolimus on KS was examined in renal-transplant recipients that exhibited cutaneous Kaposi's sarcoma. The 15 transplant patients were switched from the immunosuppressant drug cyclosporine A to sirolimus, which inhibited the progression of dermal KS lesions within three months of therapy [41]. In another clinical study, post-transplant KS patients treated with sirolimus displayed decreased KSHV viral copy number in Multicentric Castleman's disease (MCD) and PEL patients, which correlated with an enhanced CD4 and CD8 immune response against KSHV [42]. Since mTOR was recently identified to play a positive role in the regulation of KSHV replication transcriptional activator (RTA) viral gene expression, sirolimus treatment may function in KS tumor regression by blocking mTOR-mediated KSHV reactivation [43]. However, it remains unclear whether KSHV lytic replication directly contributes to tumor development *in vivo*. Although the clinical success of rapamycin treatment has been

observed in the prevention of post-transplant Kaposi's sarcoma and a potential use as an inhibitor of KSHV lytic replication, rapamycin may not be an efficacious therapy for all viral infections. Following liver transplantation, recurrent HCV infection was associated with worse poor patient and graft survival outcome [44]. Using the Scientific Registry of Transplant Recipients (SRTR) database, there was a significant association of sirolimus treatment and increased mortality and graft loss in HCV-positive liver transplant patient recipients [45]. The molecular mechanism of how sirolimus negatively affects post-transplant outcomes in HCV patients remains unclear. Further studies are necessary to determine the molecular basis of how mTOR inhibitors modulate viral infection and whether autophagy induction by mTOR inhibitors will have any anti-tumors effects on tumor virus associated cancers.

5.1 Autophagy inhibitors and their potential role in cancer treatment

Autophagy is considered to be a tumor suppressive pathway, yet autophagy upregulation can also serve as a pro-survival mechanism for certain types of tumor cells [46] [46]. Specifically, hypoxia caused by anti-angiogenic therapy induces tumor cell autophagy as a cytoprotective adaptive response, thereby promoting treatment resistance. Thus, treatment of hypoxic cells with autophagy inhibitors caused a shift from autophagic to apoptotic cell death *in vitro* [46]. Indeed, the lysosomotropic anti-malarial drugs hydroxychloroquine (HCQ) and chloroquine (CQ), in combination with anti-cancer therapies, are currently being investigated as a potential use in sensitizing tumor cells to chemotherapy and/or radiation [47,46]. HCQ and CQ indirectly function as autophagy inhibitors by blocking lysosomal acidification, thereby inhibiting the autolysosomal degradation step of autophagy. Results from current ongoing clinical trials using HCQ- and CQ-mediated autophagy modulators will determine whether autophagy inhibitors play a role in cancer treatment. Understanding the functional role of autophagy in the tumor microenvironment needs to be studied in more depth in order to determine whether response to cancer treatment is more favorable with autophagy inducers or autophagy inhibitors.

5.3 Future role for autophagy-inducing peptides as a targeted cancer therapy

Current autophagy modulator drugs in clinical use can exert pleiotropic effects, suggesting a need for the development of specific, targeted autophagic inducing compounds. A promising avenue in cancer research is the development of peptide-mediated immunotherapies that specifically trigger the autophagy pathway. The KSHV viral FLIP derived peptides Tat-vFLIP- 2 and Tat-vFLIP- 4 contain autophagic death-inducing therapeutic activity by diminishing the ability of cellular FLIP or viral FLIP to bind Atg3, leading to robust autophagy induction and autophagic cell death of KSHV-induced tumor cells *in vitro* [29]. Combinatory treatment of either vFLIP peptide with rapamycin further enhanced autophagic cell death. Using a murine xenograft PEL model, mice treated by intraperitoneal injection with the Tat-vFLIP- 2 or - 4 peptides displayed marked tumor regression in comparison to control Tat-treated mice. The *in vivo* efficacy of the vFLIP peptides as a therapeutic agent against KSHV-associated malignancies is currently being investigated using a marmoset primate model. The Tat-beclin 1 peptide was recently constructed by identifying the sequence necessary and sufficient for autophagy induction by performing mapping studies with the HIV-1 virulence factor Nef [48]. The Tat-beclin 1 peptide induces the canonical autophagy pathway by potentially promoting the release of Beclin-1 from the Golgi, thereby enhancing early autophagosome formation. In addition to inducing autophagy *in vitro* and *in vivo*, the Tat-beclin 1 peptide was associated with clearance of small polyglutamine expansion protein aggregates [48]. Since autophagy levels are decreased in hepatocellular carcinoma patients and mice with heterozygous disruption of beclin-1 exhibit a higher frequency of hepatocellular carcinoma, peptides specifically directed at increasing autophagy levels via Beclin-1 activity may serve as a therapeutic treatment of human

cancers. There is an urgent need for more effective and less toxic cancer therapies, particularly for cancers caused by human tumor viruses. The development of specific autophagy-inducing peptides, such as the vFLIP and Tat-beclin 1 peptides, represent biologically active molecules that show promising potential for use as an anti-cancer therapy.

6. Conclusions

Autophagy is an evolutionarily conserved and important homeostatic process for the degradation of cytoplasmic material. Advances in autophagy research emphasize an expanding role for autophagy in the regulation of innate and adaptive immune responses against foreign pathogens. In this review, we have highlighted recent studies on the interplay between autophagy and human tumor viruses. Certain tumor viruses utilize various strategies to antagonize the autophagy-associated host innate immune response. The gamma-herpesviruses EBV and KSHV encode viral effector proteins to escape autophagy-mediated immune programs, thus contributing to the maintenance of latent infection. In contrast, the human tumor viruses HBV and HCV positively regulate autophagy induction for the accumulation of autophagosome membranes to facilitate viral replication. However, the precise mechanisms of how viruses modulate the autophagic response during infection still remain elusive. Thus, a better understanding of the mechanistic links among cellular autophagy, immune signaling, and antiviral defense may provide therapeutic strategies and ultimately lead to discovery of novel drug targets for better treatments of cancers caused by tumor viruses.

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Figure 1. Modulation of the host autophagy pathway by human tumor viruses

The autophagy pathway functions as an intrinsic antiviral defense mechanism by directly engulfing foreign pathogens for lysosomal mediated degradation. The human tumor viruses Epstein-Barr virus (EBV), Kaposi's Sarcoma-Associated Herpesvirus (KSHV), Hepatitis C virus (HCV), and Hepatitis B virus (HBV) have evolved multiple strategies to either subvert or enhance steps in the autophagy pathway for their replication and pathogenesis. **A.** Upon autophagy initiation, Beclin-1 forms an activating complex with the class III PI3 kinase Vps34 for vesicle nucleation. The gamma-herpesviruses counteract autophagy initiation by encoding viral Bcl-2 homologs, which strongly bind to Beclin-1 to function as a repressor complex of vesicle nucleation. In contrast to gamma-herpesviruses, the HBV protein HBx stimulates autophagy induction by binding to Vps34. During vesicle elongation and autophagosome formation, LC3 undergoes lipid conjugation via an ubiquitin-like system. KSHV vFLIP blocks autophagosome formation by binding to Atg3, thereby preventing LC3 processing. HCV NS5B promotes autophagosome formation by enhancing LC3 lipid conjugation via its interaction with Atg5. HCV NS4B has been shown to complex with Vps34 and Rab5, but the mechanism of how HCV prevents the maturation of autophagosomes into autolysosomes remains unclear. Degradation of viral antigens in autolysosomes can be presented onto MHC molecules, such as the EBV EBNA1 epitope. **B.** The unfolded protein response (UPR) pathway, which is commonly activated during viral replication (EBV LMP1 and HBV HBs), can indirectly contribute to autophagy activation. Phosphorylation of the protein kinase PERK leads to downstream transcription of LC3 and

Beclin-1 via ATF4 translation, thereby contributing to autophagy induction. **C.** Acute oncogenic stress activates DNA damage responses (DDRs), ultimately triggering autophagy and oncogene-induced senescence (OIS). Expression of KSHV v-cyclin activates an oncogenic stress response and DDRs. KSHV-infected cells remain refractive to OIS by vFLIP-mediated subversion of autophagy.