REVIEW

Extracellular vesicles: novel vehicles in herpesvirus infection

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Herpesviruses are remarkable pathogens that have evolved multiple mechanisms to evade host immunity, ensuring their proliferation and egress. Among these mechanisms, herpesviruses utilize elaborate extracellular vesicles, including exosomes, for the intricate interplay between infected host and recipient cells. Herpesviruses incorporate genome expression products and direct cellular products into exosomal cargoes. These components alter the content and function of exosomes released from donor cells, thus affecting the downstream signalings of recipient cells. In this way, herpesviruses hijack exosomal pathways to ensure their survival and persistence, and exosomes are emerging as critical mediators for virus infection-associated intercellular communication and microenvironment alteration. In this review, the function and effects of exosomes in herpesvirus infection will be discussed, so that we will have a better understanding about the pathogenesis of herpesviruses.

KEYWORDS   herpesviruses; extracellular vesicles (EVs); infection; pathogenesis

INTRODUCTION

Human herpesviruses (HHVs) are distributed worldwide, and more than 90% of adults are infected by one or multiple HHVs (van Diemen and Lebbink, 2016). Once the host is attacked by these viruses and incapable of eliminating the pathogen, HHVs can establish lifelong latent infections and cause symptoms, even resulting in serious diseases such as shingles and tumors (van Diemen and Lebbink, 2016; Olsson et al., 2017). The HHV family contains three contagious sub-families: the alpha herpesvirus family [herpes simplex virus 1(HSV-1), HSV-2, and varicella-zoster virus (VZV)], beta herpesvirus family [human cytomegalovirus (HCMV), HHV6, and HHV7], and gamma herpesvirus family [Epstein-Barr virus (EBV) and Kaposi's sarcoma–associated herpesvirus (KSHV)] (Dreyfus, 2013). A seroepidemiology of HHVs was conducted using 3, 444 follow-up samples, and the results revealed that the yearly incidence of HHVs remains high (Olsson et al., 2017). Among the HHV family members, EBV and KSHV have been confirmed to be tumor viruses.

Extracellular vesicles (EVs) have recently been revealed as itinerant cargo that engage in intercellular communication and immunity regulation (Szatanek et al., 2017). There are different types of EVs, including exosomes, apoptotic bodies, microvesicles, and retrovirus-like particles (Akers et al., 2013). Because exosomes are the most widely studied type of EVs, the characterization of exosomes will be presented in more detail in this review.

Since the first evidence of EVs was presented, there have been many studies on the origin and biogenesis of EVs, in which the endosomal network is quite essential. Vesicles that are destined to form exosomes are fused with early endosomes and then shed into late endosomes [also termed multivesicular bodies (MVBs)]. During this process, endosomes sort their contents into vesicles, and late endosomes then fuse with the plasma membrane, followed by their excretion out of the cell to become exosomes (Akers et al., 2013). A Golgi component in exosomes has been confirmed (Sotelo and Porter, 1959; Lee Y et al., 2012). Although the precise mechanisms regarding the origins of exosomes remain unclear, it has been claimed that lipid rafts (which form divided microdomains), transmembrane proteins, protein complexes such as endosomal sorting complexes required for transport (ESCRT), and Rab proteins (which modulate vesicular traffic) are responsible for exosome biogenesis (Duijvesz et al., 2011; Hurley, 2015). However, conclusive reports to confirm the exact process for shaping exosomes are lacking.

Exosomes are nanoscale EVs and play critical roles in cell-to-cell communication. Exosomes contain DNA, RNA, proteins, and other bioactive molecules, allowing the exchange of genetic information and regulation of related physiological and pathological activities. Numerous studies have shown that exosomes released from tumor cells may affect tumor formation, growth, angiogenesis, metastasis, and drug resistance. Furthermore, exosomes assist tumor cells in avoiding immune surveillance and prevent the tumor niche generation (Zhang et al., 2015b; Tkach and Théry, 2016). In addition, aside from their role in tumor progression and metastasis, exosomes can protect against bacterial and viral infection, adjust tumor immunity, and mediate the immune suppression of host cells (Wang et al., 2017). Exosomes have become a popular research topic, resulting in intensive basic or clinical studies on their roles in tumor development and use as targets for drugs.

Even though the immune system employs various methods to fight and remove pathogens, viruses always evolve multiple counteracting mechanisms to evade the host immune system and allow their persistence (Anderson et al., 2016). The emerging data on microvesicles, including exosomes, represent a potent weapon in efforts to protect against viruses. When infecting host cells, viruses inject their own components into exosomes. Once released, exosomes convey their contents, affecting neighboring and distant recipient cells. Cumulatively, viral nucleic acids, such as non-coding RNAs, and proteins expressed in exosomes deceive the immune system and even influence uninfected host cells, allowing viruses to achieve permanent and even lifelong persistence (Anderson et al., 2016; Hancock and Skalsky, 2017).

Recently, David M Knipe and his colleagues summarized some viral gene products that can promote the epigenetic silencing of genomes, placing great emphasis on their potential as a promising target in latent infections (Knipe et al., 2017). Similarly, HHVs can also utilize these important exosomal vehicles to mediate their pathological process and facilitate cell-cell communication. In this review, we will provide an update on the function of exosomes in HHV infections, with the aim of advancing the knowledge on the pathogenesis and persistence of herpesvirus infection and providing novel insights into virus-host interactions.

EXOSOMES IN ALPHA HERPESVIRUS INFECTION

Alpha herpesviruses include important pathogens, such as HSV-1, HSV-2, and VZV (Hogue et al., 2016). Increasing lines of evidence indicate that alpha herpesviruses utilize exosomes to deliver cargo such as transcripts (mRNAs, miRNAs, and DNA) and proteins.

HSV-1 infects the majority of the world's population at some point in their lives. The innate immune system, including macrophages, responds to the viral attack. A research team (Miettinen et al., 2012) identified and analyzed 516 human proteins from the secretome of HSV-1-infected macrophages and found that a large proportion of the secreted proteins were intracellular proteins and nearly 80% of the proteins whose secretion increased more than 2-fold were known exosomal proteins. Moreover, their study also revealed that HSV-1-infected macrophages possibly play a role in antiviral defense, thus providing a global perspective of HSV-1. In addition, the stimulator of IFN genes (STING), a vital innate immune adaptor to HSV-1 infection, plays a critical role in the course of infection. A report demonstrated that STING as well as viral miRNAs and mRNAs are transported via exosomes from infected cells (Kalamvoki M et al., 2014). While some viral mRNAs may silence viral genes in latent infected cells, this report suggested that HSV-1 controls its virulence to ensure dissemination between individuals. This might represent a strategy used by the virus to avoid being eliminated by host cells and establish lifelong infection in balance with the host (Kalamvoki Maria and Deschamps, 2016). According to another report, two HSV-1 miRNAs termed miR-H28 and miR-H29 control viral replication and transmission from infected to uninfected cells through ectopic expression before infection. Because HSV-1 is transmitted by contact between transmitters and recipients, viral replication and transmission are requirements for the effective spread of the virus (Han et al., 2016).

It is well recognized that the envelopment and egress

of viruses are complicated processes, and HSV-1 forms mature viruses through the budding of viral glycoproteins and nucleocapsids into the lumens of cytoplasmic membranous compartments. HSV-1 was the first large assembled DNA virus to be reported as being sorted into MVBs (Crump et al., 2007). Vps4, an enzyme required for producing luminal vesicles, is essential for the cytoplasmic assembly of HSV-1. Moreover, Vps4 is also an ATPase that is required for four protein complexes that function as ESCRTs. It has been demonstrated that the dominant-negative ESCRT-III complex overwhelmingly inhibits HSV-1 replication by preventing virus envelopment, in line with the role of Vps4. The ESCRT-III complex and Vps4 are packed into mature HSV-1 virions and exosomes, whereas ALIX and TSG101, two dominant-negative proteins, are detectable at low levels and contribute to HSV-1 production (Pawliczek and Crump, 2009). Specifically, for many enveloped viruses, ESCRT provides sites for viral proteins involved in assembly and envelopment to interact intimately, whereas viruses, including HSV-1, recruit ESCRT components to specific compartments. In addition, glycoprotein B (gB, encoded by HSV-1), HLA-DR (DR) molecules, and CD63 (a late endosomal marker) are also present in exosomes. The glycoprotein of gB manipulates DR via hijacking DR from the primary transport route into the exosomal pathway and thus provides a new method of viral immune evasion (Temme et al., 2010). Moreover, the site of intracellular gB varies, indicating that gB is dependent on the exosomal pathway. The intracellular trafficking of gB and the envelopment/egress of HSV-1 rely on MVB biogenesis (Calistri et al., 2007). This further suggests that the sorting of gB into MVB membranes is obligatory for the formation of mature HSV-1 and that modified MVB membranes provide a platform for the recruitment of MVB components to allow virus envelopment and egress.

HSV-2 is one of the most prevalent genital pathogens in humans, infecting the genital tract mucosa (Lee and Ashkar, 2012; Zhu et al., 2017). HSV-2 is also involved in HIV infection and prevalence, and the ability of HSV-2 to evade the immune response and establish a latent infection has made it difficult to develop an effective vaccine (Chan et al., 2011). The innate and adaptive immune processes underlying the response to HSV-2 provide a powerful foundation for the research and development of an effective HSV-2 vaccine (Akinyi et al., 2017; Zhu et al., 2017). However, to date, there has not been a study on the relationship between HSV-2 infection and exosomes.

VZV is a highly contagious virus that only affects humans. VZV can cause chickenpox among the elderly and immunocompromised individuals during acute infection (Ahmed et al., 2015). Although primary chickenpox is typically self-limited, VZV can remain dormant in the nervous system and then is reactivated in a weak host, leading to shingles or inflammation (Kurapati et al., 2017). Given that T cells play a crucial role in disseminating VZV to the skin and ganglia in the early period of VZV infection, a nonhuman primate model of VZV infection was used to demonstrate VZV-T cell interactions. The data revealed that the gene expression of the model for regulating cell metabolism, antiviral immunity, and the cell cycle were altered (Arnold and Messaoudi, 2017). To date, no study has addressed the role of exosomes in VZV.

EXOSOMES IN BETA HERPESVIRUS INFECTION

The beta herpesvirus family contains HCMV, HHV6, and HHV7. HCMV has been widely studied; however, data on HHV6 and HHV7 remain scarce.

HCMV is a prototypical beta herpesvirus. With nearly 40 known gene products, HCMV is involved in multiple physical and pathological processes, including processing and presenting antigens, interferon and chemokine signaling, initiating apoptotic signaling, and functioning as a cytokine, to evade and subvert the host immune defense (Hudson, 2014). HCMV infection is always associated with acute allograft ejection and chronic allograft vasculopathy in transplant patients whose immune system is suppressed. By stimulating endothelial cells (ECs) to release antigenic exosomes, HCMV exacerbates allograft rejection in response to the allogeneic CD4$^+$ memory T cell-mediated immunity (Walker et al., 2009), revealing a novel manner for EC-derived exosomes to evade immune surveillance. In addition, because ESCRT machinery is important for incorporating the viral cargo into MVBs, HCMV is reported to undergo maturation independent of ESCRT components, different from HSV-1 (Fraile-Ramos et al., 2007). However, another study reported that the final step in HCMV production requires the ESCRT machinery (Tandon et al., 2009). Thus, the exact site of the final envelopment for HCMV remains unclear and warrants further investigation. Based on the study by Cepeda V *et al.*, HCMV generates vesicles that traffic between the trans-Golgi network (TGN) and endosomes. The vesicles include the TGN and endosome markers (TGN46, annexin I, CD63, endosomal marker early endosome antigen 1, transferrin receptor, and the cation-independent mannose 6-phosphate receptor) to achieve viral envelopment (Cepeda et al., 2010). Nevertheless, the mechanisms involved in virus envelopment and egress still await detailed elucidation.

Closely related to HCMV, HHV-6 is a notable beta herpesvirus and can cause primary infection or be activated from latency in liver transplant recipients (Parra et al., 2017). HHV-6 is divided into two distinct species:

HHV-6A and HHV-6B. Markedly different from other HHVs, HHV-6 integrates its genomic DNA into the sub-telomeric part of cell chromosomes in approximately 1% of the population (Agut et al., 2015). Evidence of its exosomal presence was presented in a study by Yasuko Mori *et al*. The authors showed that cells that were infected with HHV-6 were larger than uninfected ones and contained MVBs in the late period of infection. As noted above, exosomes are derived from MVBs and eventually fuse with the plasma membrane. MVBs contain CD63, which is an MVB late endosome marker and envelope glycoprotein, that is localized in internal vesicles. The available evidence indicates that HHV-6 virions are released through the exosomal pathway, which demonstrates the significance of exosomes in the HHV6 maturation pathway (Mori et al., 2008). Additionally, MHC class I molecules are another rare member that have been found to be inserted into viral particles and exosomes and then released into the extracellular space via an exosomal secretary pathway, which can be blocked by the U24 protein of HHV-6 (Sullivan and Coscoy, 2010; Ota et al., 2014). Because HHV-6 mainly replicates in T lymphocytes, MHC class I molecules are colocalized with the gB protein and incorporated into exosomes and modify HHV-6 infection (Ota et al., 2014). However, additional details about the role of exosomes in HHV-6 infection need to be addressed in future studies.

HHV-7 infection commonly occurs in infants or during childhood and may cause symptoms in cases of immunodeficiency after reactivation from a latent infection (Parra et al., 2017; Riva et al., 2017). Nevertheless, the pathogenic role of HHV-7 for non-immunosuppressed adults in diseases of the central nervous system still needs to be explored, and HHV-7 is possibly an etiological agent of suspected infectious encephalitis in individuals who do not respond to acyclovir (Parra et al., 2017). There is no published report on the roles of exosomes in HHV-7 infection.

EXOSOMES IN GAMMA HERPESVIRUS INFECTION

The gamma herpesvirus family is made up of EBV and KSHV, both of which are closely associated with multitudinous malignancies. Even though EBV and KSHV were originally observed in human tumor specimens in 1964 and 1994, respectively, the finding that a portion of the human population was infected by both EBV and KSHV received great attention (Pegtel, 2013). When the two herpesviruses combine in aggressive lymphoma, primary effusion lymphoma (PEL), they can bring about an extraordinary synergic effect on lymphomagenesis, leading to increased complications (Meckes et al., 2013; Choi et al., 2017). A large number of investigations have revealed that these two viruses share similar colonization and persistence strategies, such as deregulating NF-κB to control B-cell proliferation and secreting proinflammatory mediators that contribute to tumorigenesis. Exosomes are another key strategy for the virus-associated tumor cells to facilitate the development of an adaptive tumor microenvironment that shapes the tumor (Pegtel, 2013). To confirm the effects of EBV and KSHV on exosomal content, proteomics techniques, including mass spectrometry, were performed on exosomes derived from cell lines that were uninfected, infected with alternative, or infected with both EBV and KSHV. The results indicated that exosomes from cells infected by these two viruses probably regulate cell death and survival, protein synthesis, ribosome function, and some signaling pathways. EBV-related exosomes mainly influence cellular signalings such as NF-κB, IFN, integrins, and actin, whereas KSHV-related exosomes prevailingly impact cell metabolism (Meckes et al., 2013). Gamma herpesviruses alter metabolic phenotypes to allow viral infection and everlasting persistence, and Angela Kwok-Fung Lo *et al*. speculated that this modulation is conductive to tumor progression, suggesting a new opportunity for therapeutic intervention (Lo et al., 2017).

EBV was the first human tumor virus confirmed to be associated with diverse malignancies, including nasopharyngeal carcinoma (NPC), Hodgkin's lymphoma, Burkitt's lymphoma, and gastric cancer. In our work on viral pathogenesis, the copy number of the EBV latent genome was found to correlate strongly with oncogenicity (Zuo et al., 2015). Emerging lines of evidence demonstrate the increasing functions and roles that exosomes have in the EBV tumorigenic process. Latent membrane protein 1 (LMP1) is the oncoprotein of EBV and is considered a key modulator in the pathogenesis of NPC. LMP1 causes the disfunction of signal transduction pathways such as NF-KB, STAT, and JAK3 (Zheng et al., 2007). Elevated telomerase activity is ubiquitous among individuals with cancers. LMP1 modulates telomerase activity through the p16INK4A/Rb/E2F1 and JNK signaling pathways in NPC (Ding et al., 2010). Moreover, LMP1 can also upregulate MDM2 protein, which is a cellular pro-oncogene that is frequently abnormally overexpressed in NPC, and lead to the initial vast accumulation of ubiquitinated MDM2 (Li et al., 2007). EBV traffics LMP1 into exosomes, altering the contents and function of exosomes. LMP1-modified exosomes promote the growth, migration, and invasion of cancer cells, and a tetraspanin protein, CD63, aids in the incorporation of LMP1 into exosomes. Moreover, CD63 is a critical partner of LMP1 to enhance exosome production and limit the activation of downstream signaling pathways such as NF-κB (Hurwitz et al., 2016). In addition, EBV-infected cells influence recipient cells and the surrounding microenvironment through exosomal miRNAs, Fas

ligand, EBERs and other non-coding RNAs (Ahmed et al., 2015; Dolcetti, 2015; Iwakiri, 2015; Baglio et al., 2016; Gallo et al., 2016; Lin et al., 2016; Yoon et al., 2016; Zuo et al., 2017). MiRNAs are regarded as essential mediators in a variety of processes by orchestrating distinct targets and molecular pathways, and they are involved in immunological activities such as congenital immunity and virus infection (Li et al., 2010; Jia et al., 2014). EBV encodes its own miRNA and dysregulates host cellular miRNAs (Zuo et al., 2017; Yu et al., 2012). The miRNAs upregulated by EBV in cells are easily packaged into exosomes. Because miRNAs are usually enriched in exosomes, exosomal miRNAs are potential biomarkers of cancers and other diseases. Our recent work also showed that EBV-dysregulated long noncoding RNAs and cellular proteins may be transmitted to neighboring or distant cells (will be published elsewhere). This is a notable viral pathogenesis mechanism. In short, viruses hijack host exosomal pathways, and virally modified exosomes may accelerate the immune evasion, proliferation, and spread of the viruses (Meckes, 2015). Regarding virus-associated carcinoma, changes in exosome biology may contribute to tumor formation, development, metastasis, and invasion. Exosomes associated with EBV might be another possible approach to halt the progression of cancer besides the method of chemical agents like lactoferrin (Zheng et al., 2012).

KSHV is one of the multiplex carcinogenic viruses that are able to induce tumors in immunocompromised individuals. KSHV participates in the progression of Kaposi's sarcoma, HIV in immune-suppressed patients, and other pathogens (Thakker and Verma, 2016). KSHV establishes lifetime persistence and exhibits two different life cycles: a prolonged latent state and a short productive or lytic life cycle. In the former period, the virus integrates its episome into the host chromosome and is replicated during cell division. Latency-associated nuclear antigen (LANA), a multifunctional nuclear protein of KSHV, is expressed during latency. Pravinkumar Purushothaman proved that LANA has the capacity to modulate a diverse array of infections (Purushothaman et al., 2016). There are few available models for KSHV, but in a mouse model of KSHV and in patients with KSHV-associated malignancies, exosomal miRNAs of the host and those encoded by KSHV, including the miR-17-92 cluster, were successfully detected. Furthermore, the characterization and analysis of exosomal miRNAs, their targets, and endothelial cells revealed that exosomes containing a wide variety of miRNAs play a functional biological role in signaling pathways linked to the pathogenesis of KSHV and its phenotypic characteristics (Chugh et al., 2013; Zhang et al., 2015a). Hence, distinct exosomal miRNAs are expected to represent a portion of the paracrine signaling mechanism, which is the hallmark of KSHV tumorigenesis. Aside from miRNAs, gamma interferon-inducible protein 16 (IFI16), a nuclear pathogen sensor, and cleaved interleukin-1β (IL-1β), derived from the exosomes of B lymphoma cells, can be detected (Chugh et al., 2013). KSHV was found to utilize a strategy that disrupts the innate defense against inflammation. Indeed, due to the complexity of viral attachment and entry, KSHV enters cells by dynamin-dependent clathrin-mediated endocytosis and dynamin-independent micropinocytosis. It was revealed that irregular cup-shaped endocytic vesicles are required for successful infection (Veettil et al., 2014). However, no other prominent study on exosomes related to KSHV has been published to date.

CONCLUSIONS

Herpesviruses are remarkable pathogens that efficiently utilize host components and resources to evade host immunity and achieve proliferation and egress. Although

Table 1. Exosomal molecules utilized by herpesviruses during infection

Sub-families	Classification	Molecules related to exosomes
Alpha herpesvirus	HSV-1	STING, miR-H28, miR-H29, ESCRT-III complex, Vps4, ALIX, TSG101, gB, DR, CD63
	HSV-2	none at present
	VZV	none at present
Beta herpesvirus	HCMV	TGN 46 and endosomes markers (annexin I, CD63, endosomal markers early endosomal antigen 1, transferrin receptor, and the cation-independent mannose 6-phosphate receptor)
	HHV-6	CD63, MHC class I molecules, gB
	HHV-7	none at present
Gamma herpesvirus	EBV	LMP1, CD63, miRNAs, Fas ligand, EBERs, and other non-coding RNAs, NF-κB, IFN, integrins, and actin
	KSHV	IFI16, IL-1β

the mechanism related to EVs or exosomes utilized by herpesviruses has not been completely elucidated, available evidence suggests an elaborate process by which herpesviruses promote the interplay between infected and recipient cells. Herpesviruses inject genome expression products of their own and usurp cellular signaling molecules in exosome cargoes (Table 1). Viruses such as HSV-1 can even be packaged into MVBs, which have a similar size as EVs. These exosomal components function in downstream signaling. Therefore, exosomes not only can serve as a biomarker for the diagnoses of various diseases, including virus infections, but also could be targets used for the prediction and therapy of tumors or related diseases.

With the increasing number of studies on exosomes in virus infections, many issues need to be explored, including how the host exosomal proteins react to the virus infection, the molecular mechanism by which exosomes affect the envelopment and assembly of virions, and how herpesviruses assemble and egress with exosomes. The exploration of these questions will lay a better foundation for the development of vaccines and help obtain a deeper understanding of the pathogenesis of herpesvirus infection.

ABBREVIATIONS

HHVs, human herpesviruses; HSV-1, herpes simplex virus 1; HSV2, herpes simplex virus 2; VZV, varicella-zoster virus; HCMV, human cytomegalovirus; EBV, Epstein-Barr virus; KSHV, Kaposi's sarcoma–associated herpesvirus; STING, the stimulator of IFN genes; Vps4, an enzyme required for producing luminal vesicles; MVB, multivesicular body; ESCRTs, the function of endosomal sorting complexes required for transport; ALIX and TSG101, two dominant-negative proteins; gB, HSV-1 encoded glycoprotein B; DR, HLA-DR; CD63, the late endosomal marker; EC, endothelial cells; TGN, the trans-Golgi network; U24, a protein of HHV-6; PEL, primary effusion lymphoma; NPC, nasopharyngeal carcinoma; LMP1, latent membrane protein 1; MDM2, a cellular pro-oncogene protein; LANA, Latency-associated nuclear antigen of KSHV; IFI16, gamma interferon-inducible protein 16; IL-1β, interleukin-1β.

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