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Coxsackievirus B transmission and possible new roles for extracellular vesicles

Jameel M. Inal[1] and Samireh Jorfi

Cellular and Molecular Immunology Research Centre, School of Human Sciences, London Metropolitan University, 166–220 Holloway Road, London N7 8DB, U.K.

Abstract

Coxsackievirus B1, a member of the *Picornaviridae* family is a non-enveloped single-stranded RNA virus associated with human diseases including myocarditis and pancreatitis. Infection of the intestinal mucosa, lined by polarized epithelial cells, requires interaction of coxsackievirus with apically located DAF (decay-accelerating factor) before transport to the basolaterally located CAR (coxsackie and adenovirus receptor), where entry is mediated by endocytosis. As with many other non-enveloped viruses, coxsackievirus has to induce lysis of host cells in order to perpetuate infection. However, recent evidence indicates that virus spread to secondary sites is not only achieved by a lytic mechanism and a non-lytic cell–cell strategy has been suggested for coxsackievirus B3. A physical interaction between infected and non-infected cells has been shown to be an efficient mechanism for retroviral transmission and one type of extracellular vesicle, the exosome, has been implicated in HIV-1 transmission. HIV-1 also takes advantage of depolymerization of actin for spread between T-cells. Calpain-mediated depolymerization of the actin cytoskeleton, as a result of increases in intracellular calcium concentration during coxsackievirus infection, would result in a release of host cell-derived microvesicles. If so, we speculate that maybe such microvesicles, increasingly recognized as major vehicles mediating intercellular communication, could play a role in the intercellular transmission of non-enveloped viruses.

Introduction

MVs (microvesicles) and exosomes are together known as EVs (extracellular vesicles). Their release is an important mechanism by which cells communicate with each other. Platelets, for example, release MVs which transfer tissue factor to monocytes, thus allowing monocytes to participate in the coagulation pathway [1], and MV release itself, as we reported previously [2], is a means of unconventional protein export for proteins lacking an N-terminal sequence. Despite these essential functions of MV release for (host) cells, it is now clear that some intracellular pathogens exploit this mechanism for their infection. Notably, we demonstrated recently that *Trypanosoma cruzi* metacyclics evade host complement-mediated lysis by fusing with blood-cell-derived MVs, which inhibit complement activation by accelerating decay of the C3 convertase [3]. We have also shown that interaction between *T. cruzi* metacyclics and host cell receptors results in MV production from the host cell plasma membrane which the parasite exploits for invasion [4].

Microvesiculation can be induced by a plethora of cellular events including cell death, hypoxia, stress, expression of oncogenes, differentiation and infection by viruses [5]. With the observation that ESCRT (endosomal sorting complex required for transport) components are recruited to the site of budding, a hypothesis arose postulating that retroviruses have adapted to use host exosome machinery for the formation and transfer of virions by a non-viral route [6]. The similarities of biogenesis of enveloped viruses such as retroviruses and exosomes thus resulted in the postulation of the Trojan exosome hypothesis relating to HIV assembly and cell–cell spread. However, ceramide inhibition (which halts exosome shedding) had no effect on HIV budding, in spite of a decrease in infection [7–9], confirming that exosomes are not solely involved in HIV budding from the plasma membrane.

Similar to most non-enveloped single-stranded RNA viruses, CVB1 is well established to cause infection via the lytic mode of infection, which requires contact between the virus and the target cell plasma membrane [10]. However, although recent reports have described several non-lytic virus release mechanisms by which virus particles are transmitted to secondary sites of infection, these processes remain unclear [11], and thus constitute the main theme of the present mini-review.

EVs and viral infection

The use of exosome machinery for biogenesis and spread [12] is widespread among enveloped viruses including rhabdoviruses, filoviruses, arenaviruses, herpesviruses, and hepatitis B and C viruses. More intensive research will be required, however, to establish whether inhibition of exosomes and virus release could become a reliable future target to tackle infection. A technical problem in this field has until recently been that exosomes have almost the same size and density as viruses, making it a challenge to study

Key words: coxsackievirus, exosome, infection, microvesicle, transmission.

Abbreviations used: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CAR, coxsackie and adenovirus receptor; CVB, coxsackievirus B; DAF, decay-accelerating factor; EBV, Epstein–Barr virus; EV, extracellular vesicle; miRNA, microRNA; MV, microvesicle; Nef, negative regulatory factor protein.

[1]To whom correspondence should be addressed (email j.inal@londonmet.ac.uk).

secreted exosomes during viral infection [9]. However, new purification methods have been developed using iodixanol density gradients and immunoaffinity isolation, which has led to the separation of pure exosomes from HIV virions [13].

Exosomes play important roles in HIV infection. They may carry co-receptors for HIV between cells, thus helping viral infection of target cells. Exosomes expressing Nef (negative regulatory factor protein) of HIV are also able to induce apoptosis of $CD4^+$ T-cells. Such vesicular transfer of Nef to target cells [14] provides a significant means by which exosomes evade the immune system. However, infected cells shed viral-antigen-bearing exosomes that may activate innate immune responses [15]. Overall, the similarities between enveloped viruses and exosome biogenesis have contributed to our understanding of exosome biology [16].

CVB (coxsackievirus B) 1 is a member of the *Picornaviridae* family, which are among the most common human pathogens and include coxsackieviruses, PV (poliovirus), HRVs (human rhinoviruses), FMDVs (foot-and-mouth disease viruses) and HAV (hepatitis A virus) [17]. CVB1 is a non-enveloped single-stranded RNA virus associated with a broad spectrum of human diseases including myocarditis, meningoencephalitis, pancreatitis and paralytic myelitis and the six CVB serotypes are each responsible for different symptoms and diseases. It has been reported that picornaviruses are able to depolymerize the host cytoskeleton during infection so aiding the spread of virus; this disruption of the actin cytoskeleton also leads to release of MVs [18].

Mechanisms of viral entry into cells

In order to infect, viruses confront a number of barriers to entry such as the glycocalyx which blocks virus access to the cell surface. Polarized epithelial cells locate receptor molecules to the basolateral cell surface where they are not accessible to viruses, and specialized cellular junctions make the epithelium impermeable [19]. The formation of the virus–cell receptor complex results in activation of cellular signalling cascades and ligand-triggered processes such as calveolar/raft endocyosis, clathrin coat assembly and actin cortex dissociation [20]. Viruses only infect cells with specific viral receptors, although many viruses are known to use more than one type of receptor, either in parallel or in series. For example, HIV-1 binds to glycosylceramides and heparan sulfate, interactions that may facilitate the initial recruitment of virus to susceptible cells [21].

Furthermore, the presence of specific glycosphingolipids in the target cell membrane can enhance CD4/co-receptor-dependent fusion [22]. Some viruses undergo rapid mutation and may switch receptors or even use alternative receptors which, in the case of avian influenza, may result in a greatly increased risk that the virus may interact with glycoconjugates on human cells. The release of viral genome into the cytosol is called penetration [23], and non-enveloped viruses use a pore-formation mechanism to release their genome in contrast with enveloped viruses which use membrane fusion [24].

CVB and its six serotypes together with rhinovirus and encephamyocarditis virus (also members of the *Picornaviridae* family) use CAR (coxsackie and adenovirus receptor) for cell entry [25] and adenoviruses share the same receptor for cell entry. CAR is a 45 kDa glycosylated transmembrane receptor with a 107-amino-acid cytoplasmic domain, and two extracellular immunoglobulin domains (D1 and D2). CAR is a member of the family of intercellular adhesion molecules that include intercellular adhesion molecule and vascular cell adhesion molecule receptors and functions in both attachment and infection of CVB. In adenovirus infection, CAR is only involved in attachment and integrins are associated with the entry of virus into cells [26]. Before the interaction with CAR, CVBs use the GPI (glycosylphosphatidylinositol)-anchored complement receptor protein, DAF (decay-accelerating factor), and adenoviruses use integrins as co-receptors to infect [23].

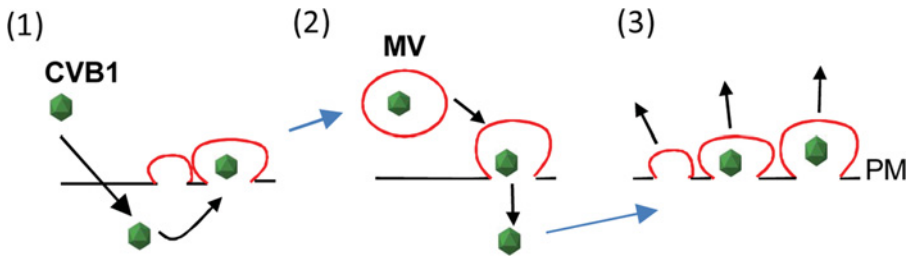
As in epithelial cells, CAR is located at tight junctions, protein complexes that are in charge of the selective passage of ions and molecules across the epithelium and therefore not accessible to incoming viruses from the apical side, CVBs must first interact with the DAF co-receptors present on the apical surface. Virus binding leads to cross-linking of DAF molecules and activation of the tyrosine kinase c-Abl which triggers Rac-dependent actin rearrangements, permitting virus movement to the tight junction. CVBs then induce a lateral movement along the membrane and bind to filopodia to reach the CAR, which induces a conformational change in the virus particle, uncoating and entry [18]. The actin cytoskeleton and c-Abl activity are required for CVB entry and infection [27]. Upon entry, virus releases its RNA through caveolar endocytosis, which is activated by phosphorylation of Tyr^{14} in caveolin-1. CVB replicates in the cytosol and does not need low pH to penetrate [28].

The level of CAR expression is an indicator of susceptibility to viral infection in young children. Upon virus entry, the immune response is to eliminate the virus, otherwise chronic myocarditis can evolve [23], and cytokines are important mediators of the innate immune system in response to infection. Interferons comprise two types: type I, including interferon-α and -β, and type II consisting of interferon-γ. Both types of inteferon inhibit viral replication *in vitro.* In addition, upon virus entry, single-stranded RNA is released and is used as a template for the viral genome to replicate [29].

Earlier reports have described CVB1-induced apoptosis as a viral mechanism to aid maximum virus dissemination [19]. Although the exact process(es) involved remain unclear, some studies have suggested that CVB1 induction of apoptosis in neighbouring cells is not exclusively caused by the lytic escape of enteroviruses [30,31]. In addition, earlier studies have postulated a direct cell–cell spread of the poliovirus in the central nervous system [32], and a recent study has also described a non-lytic viral mechanism of cell–cell transmission that involves CVB3 induction of cellular protrusions [33].

Figure 1 | **CVB1 uses a cell–MV–cell mode of transmission**

(1) Infecting coxsackievirus stimulates the release of apoptotic MVs which are able to transmit virus (2) and to then induce apoptosis and stimulate a further release of MVs (3) from recipient cells.



Whereas enveloped viruses are released through budding from infected cells, non-enveloped viruses use cell lysis for release. It has been documented that some non-enveloped viruses, however, after budding through membrane compartments, lose their membrane or that others still gain access to exocytic organelles and are released [29]. Exosomes have a crucial role in dissemination of viruses and spread of infection and HIV manipulates host exosomes to propagate infection [34]. EBV (Epstein–Barr virus)-positive B-cells release exosomes containing viral miRNAs (microRNAs) which are transferrable to monocytes. In addition, non-infected B-cells were positive for EBV miRNAs [35] and exosomes have the potential to transfer viral genetic factors to non-infected cells [36]. Indeed, a previous study has shown that, in infectious prion diseases such as Kuru, exosomes carry the prion protein scrapie (PrP^{Sc}) and deliver it into non-infected cells, which shows a possible contribution of exosomes in prion propagation [37].

A possible role for MVs in the transmission of non-enveloped viruses such as CVB1

Many infectious pathogens have developed strategies to subvert host epithelia or endothelial barriers in order to invade and spread to secondary sites of infection. It has been established that CVBs enter polarized cells by an endocytic mechanism that requires the activation of specific signalling molecules including the Src family of tyrosine kinases [20]. Although non-enveloped viruses such as reoviruses have been shown to cause damage through the direct effect of viral infection of myocardiocytes [31], it is well established that the enhanced spread of viral progeny to secondary sites during infection is not exclusively achieved via the lytic mechanism of infection [30,31]. Although the exact mechanisms involved remain unclear, a previous study demonstrated a non-lytic cell–cell strategy that involves CVB3 induction of cellular protrusions [33]. Nevertheless, to date, there are very few data supporting a non-lytic mechanism of viral spread. Furthermore, although these data suggest a non-lytic cell–cell mechanism, it remains unclear how viral progeny disseminate to more distant regions to infect new cells.

CVBs have been reported to specifically exploit Ca^{2+}-mediated signalling events in order to facilitate their entry into polarized endothelial cells [38]. Induction of apoptosis has also been reported to be an important step during the infection process, and was shown to elicit an increase of $[Ca^{2+}]_i$ (intracellular Ca^{2+} concentration) before virus entry. Interestingly, an increase in $[Ca^{2+}]_i$ also results in calpain-mediated depolymerization of the host actin cytoskeleton and release of MVs [20].

Studies with retroviruses have shown that the physical interaction between infected and uninfected cells provide a more efficient way (by two to three orders of magnitude) for the virus to reach new targets compared with the lytic spread of infection [34,39]. Moreover, HIV-1 exploits depolymerization of the host actin cytoskeleton for intercellular spread between T-cells [40], thus suggesting a possible role for MVs in HIV infection. We speculate that there might be a non-lytic mechanism of infection involving MVs (Figure 1), by which CVB1 virions are encapsulated within MVs and disseminated to secondary sites to infect new cells.

Conclusions and future perspectives

Viral dissemination via the MV–cell mechanism may therefore offer a unique advantage to the virus, as opposed to transmission using the cell–cell strategy. As with viruses inside cells, viral progeny inside MVs would also be protected against host-mediated immune responses. MVs in viral transmission, as opposed to the lytic spread of infection, would explain the rapid dissemination of virus particles to secondary sites of infection, and the enhanced induction of apoptosis observed in neighbouring cells during CVB1 infection. Finally, because of to their size (0.1–1 μm in diameter), MVs are likely to disseminate further to distant regions and to evade immune attack, thus bringing viruses closer to several cell types to initiate viral infection.

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