VIRUS-INDUCED FORMATION OF EXTRACELLULAR MICROVESICLES DURING COXSACKIEVIRUS DISSEMINATION IN THE HOST

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Virus-Induced Formation of Extracellular Microvesicles during Coxsackievirus

Dissemination in the Host

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ABSTRACT OF THE THESIS

Virus-Induced Formation of Extracellular Microvesicles during Coxsackievirus Dissemination in the Host
by
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Coxsackievirus (CVB) is a relatively common enterovirus belonging to the picornaviridae family that is capable of causing aseptic meningitis, pancreatitis and myocarditis in humans. Coxsackievirus B3 (CVB3) preferentially targets neural progenitor and stem cells (NPSCs) with lasting consequences in the central nervous system (CNS). We genetically engineered a recombinant coxsackievirus expressing “fluorescent timer” protein (Timer-CVB3). “Fluorescent timer” protein undergoes a slow conversion from green to red over time, and thus, Timer-CVB3 can be utilized to detect the progression of virus infection. Upon infection with Timer-CVB3, partially differentiated neural progenitor and stem cells (NPSCs) and C2C12 myoblast cells released extracellular microvesicles (EMVs) containing matured “fluorescent timer” protein and infectious virus. Purified EMVs isolated from the supernatants of Timer-CVB3 infected cells were positive for the exosomal marker - flotillin-1, the autophagosomal marker - LC3-II, and viral capsid protein - VP-1. EMVs isolated by iodixanol isopycnic density purification were found in low density fractions consistent with membrane association distinct from high density viral fractions representing infectious virions. Detection of the lipidated form of LC3 protein (LC3-II) in EMVs observed within infected cell culture supernatants suggests involvement of the autophagy pathway during the release of EMVs. Also, viral protein co-localized with LC3 protein within EMVs identified in infected cultured cells, indicating that autophagy is an important process involved in release of shed microvesicles harboring infectious CVB3. Clarifying the role of these infectious EMVs is important for understanding virus immune evasion and for the development of new antiviral therapies and vaccines.
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CHAPTER 1

INTRODUCTION

1.1 SIGNIFICANCE OF COXSACKIEVIRUS

Coxsackievirus is a member of the Enterovirus genus and belongs to the Picornaviridae family. Enteroviruses (EV) are among the most medically important human pathogens. EVs are a frequent cause of central nervous system (CNS) and heart diseases. Antibodies specific to EV have been detected in 75% of individuals within developed countries [1]. Coxsackieviruses (CV) are human pathogens that cause severe mortality, particularly in infants and are transmitted through the fecal oral route. Coxsackieviruses are distinguished from other enteroviruses by their higher pathogenicity for suckling than for adult mice. Infants infected with CVB3 have been shown to be extremely susceptible to myocarditis, meningitis and encephalitis. Based on lesion formation in mice, coxsackieviruses are divided into group A and group B viruses [2]. Coxsackievirus B3 (CVB3) is a small, positive stranded RNA virus, and the viral genome encodes a single polyprotein from an internal ribosomal entry site within the 5’ untranslated region. The polyprotein is then cleaved post-translationally into eleven different proteins that include: four structural proteins (VP1-VP4), which includes the viral capsid protein and seven non-structural proteins (2A-2C and 3A-3D), which includes the RNA dependent RNA polymerase [3]. Viral protein 2B increases membrane permeability, 3A protein inhibits the intracellular transport, and viral protein 2A directs the host shut-down of cap-dependent translation. Precursors of non-structural proteins 2BC and 3A have been shown to induce the formation of double membrane autophagy-like vesicles in the absence of viral infection [4]. Previously, our lab has shown that CVB3 preferentially targets neural progenitor and stem cells (NPSCs) in the brain [5-7]. CVB3 infection may lead to long-term consequences in the CNS [8], and thus NPSCs may be a potential site for virus persistence in mice infected shortly after birth [9, 10].
1.2 RECOMBINANT CVB3

In order to track virus dissemination in vitro and in vivo, our laboratory has genetically engineered a recombinant coxsackievirus that expresses marker proteins such as eGFP and dsRED. These recombinant CVB3s have permitted tracking of CVB3 infection in live cultures. Our laboratory also developed a recombinant coxsackievirus that expresses “fluorescent timer” protein (Timer-CVB3) [11] which assists in distinguishing a newly infected cell from a previously-infected cell. The engineered mutations in the “fluorescent timer” protein extend green fluorescence for approximately 24 hours after translation due to a delayed autocatalysis process. However over a period of ~48 hours, “fluorescent timer” protein fluoresces red due to a conformational change. This progressive property of changing color in time gave us dynamic information about the progression of infection. During infection of progenitor cells, extracellular microvesicles (EMVs) expressing the viral red fluorescent protein were observed [11] in supernatants.

1.3 VIRAL INTERFERENCE WITH ENDOCYTIC PATHWAYS

Upon infection of partially differentiated neural progenitor and stem cells (NPSCs) and C2C12 myoblast cells, I observed the release of EMVs, expressing matured “fluorescent timer” protein. In recent studies, microvesicles including exosomes have been known to carry mRNA, cDNA and non-coding sequences in addition to proteins and lipids. Once released, these microvesicles can be engulfed by neighboring cells. Shed microvesicles have been found in blood (serum and plasma), urine, breast milk, saliva ascitis fluid and cerebral spinal fluid [12]. Two major mechanisms of microvesicle release have been described: exosomes and shed microvesicles. Exosomes are intraluminal vesicles (ILVs) derived from the endosomal multivesicular bodies (MVBs) and range in size between 40-100nm in size. Exosomes are either targeted to the lysosomes for degradation or are released into the extracellular milieu by fusion with the plasma membrane. In contrast, shed microvesicles are released from the plasma membrane and range in size from 100nm to 1µm [13]. Structurally, animal viruses have been categorized by the presence or absence of an envelope made of a lipid bilayer membrane. This property affects the stability, transmission and immune recognition of the virions. Picornaviruses are non-enveloped, and hence, prone to immune recognition once they exit the intracellular milieu. Recently, hepatitis A virus, also a
picornavirus, has been shown to acquire a host membrane derived by hijacking the ESCRT pathway - endosomal sorting complex required for transport, as the virus exits the host cell [14]. These results suggest that non-enveloped viruses may have developed mechanisms to egress from infected cells, become encapsulated in host membranes, and survive in the extracellular environment by evading the host neutralizing antibody response. The microvesicles that I purified from infected NPSCs and C2C12 cells included host proteins, flotillin-1 and LC3-II. Flotillin-1 co-localizes with lipid rafts on the plasma membrane, and has been found in compartments of endocytic and autophagic pathways such as late endosomes and the golgi complex [15, 16]. LC3-II is a protein essential for the formation of double membrane autophagosomes in the cell [17]. Since this marker was detected in purified microvesicles isolated from CVB3-infected progenitor cells, I hypothesized that these EMVs are derived from the autophagic pathway.

1.4 VIRAL INTERFERENCE WITH THE AUTOPHAGIC PATHWAY

Autophagy is a homeostatic process involved in the degradation of damaged cellular organelles and proteins and is generally induced by starvation. Autophagy plays a role in initiation of innate and adaptive immune responses to bacterial and viral infections and also has intracellular antimicrobial properties. Atg proteins comprise the major machinery involved in the formation of the autophagic membranes. Autophagy, represented by the mammalian target of rapamycin (mTOR) can be activated by cellular stress, ER stress, growth factor depletion, or high cell densities. Following cellular stress, mTOR is inhibited and a complex of Atg13/ULK1/FIP200/Atg101 is formed to initiate the nucleation of a phagophore. This phagophore then elongates and encloses cytoplasmic components in a double membrane vacuole, the autophagosome. The elongation process requires the formation of Atg5/Atg12/Atg16 complex and conjugate phosphatidylethanolamine (PE) into microtubule associated protein light chain 3 (LC3). The lipid chain of LC3 is involved in the formation of autophagosome. Autophagosomes fuse with lysosomes to form autophagolysosomes, wherein the engulfed material is degraded [18-20]. Many RNA viruses (such as poliovirus, rhinovirus, hepatitis A and coxsackievirus), DNA viruses (hepatitis B and parvovirus), coronaviruses and flaviviruses have been shown to induce the accumulation of autophagosomes or autophagolysosomes [4, 21-24].
Other picornaviruses, such as rotavirus have recently been shown to hijack the autophagy membranes to transport viral proteins to sites of viral replication for assembly of infectious virions and inhibition of autophagy blocks virus production [25]. CVB3 infection has been shown to induce the formation of autophagosomes without undergoing further protein degradation by fusing with the lysosome [22]. In contrast to results observed for a cardiac tumor cell line (HL-1 cells), no change in the in level of cellular autophagy in undifferentiated neurospheres infected with CVB3. In addition, a decrease in cellular autophagy was observed in partially differentiated NPSCs infected with CVB3 [26]. This suggests that viral infection in progenitor cells, and the differentiation process might alter the autophagic during CVB3 infection. A decrease in the level of LC3 puncta in differentiated NPSCs [26] suggested to us that these vesicles may be released outside the cell. Taken together, these studies indicate that hijacking the autophagic process may provide a way for a cytolytic virus like CVB3 to persist in the host and continue the release of viral progeny by exocytosis without cytopathic effects [22, 27]. I hypothesized that EMVs released from infected NPSCs and C2C12 cells are autophagosomes harboring viral material. The virus may use this novel mechanism to disseminate in the host by evading the host immune system and persist in the host. Therefore, characterizing the generation of EMVs may be important in designing novel therapeutic drugs to fight enteroviral infection.
CHAPTER 2

MATERIALS AND METHODS

2.1 GENERATION OF RECOMBINANT CVB3

The generation of recombinant Coxsackievirus expressing GFP, dsRED or “fluorescent timer” protein (eGFP-CVB3, dsRED-CVB3 and Timer-CVB3) has been previously described [28, 29]. In short, an infectious CVB plasmid (pH3) was isolated from heart of an acute myocarditis patient. This clone was engineered to contain a unique Sfi site (pMKS1) which aids in the insertion of any desired foreign sequence into the CVB3 genome. The recombinant constructs were then transfected into HeLa RW cells and supernatants containing the viral stocks were collected for titration by plaque assay.

2.2 PLAQUE ASSAY

Viral titrations by plaque assay were carried out as described before [28]. In short, HeLa cells were platted at a concentration of 1*10^5 cells/ml in a 6 well plate. Each well received a total volume of 3 mls. The plate was incubated at 37°C and 5% CO₂ overnight. The next day, each viral sample was serially diluted and the cells were infected for an hour. Each well was then filled with 4 ml of 1.2% Agar, 2X DMEM, 2.5% FBS and 1X Penicillin/Streptomycin (P/S). After the agar overlay, the plate was incubated for 48 hours at 37°C and 5% CO₂. The cells were then fixed with 75% Methanol and 25% Acetic Acid for 20 minutes at room temperature. To stain the cells, 1 ml of 0.25% crystal violet was added to every well. After staining overnight at room temperature, plaques were counted.

2.3 CELL CULTURE AND INFECTION

In this chapter, I will go over cell culture techniques and infection of various cells.

2.3.1 Neural Progenitor and Stem Cells (NPSCs)

Neurospheres were isolated and cultured as described previously (5). They were plated at a concentration of 1*10^5 cells/ml in gelatin/ fibronectin coated 6-well plates or alternatively in a µ dish (iBidi, Inc., cat#81156) containing differentiation media. For the 6-
well plates, 3 ml of media was added per well and 2 ml of media was added to the µ dish. After partially differentiating for five days, neurospheres were infected using different recombinant viruses with an MOI of 0.1. The infection was tracked at the specified time points using Zeiss Axio Observer D.1 inverted fluorescent scope.

2.3.2 C2C12 Myoblast Cells

C2C12 cells were grown in complete media (DMEM 1X+ 10% FBS+1% Anti-anti). Cells were plated at a concentration of 1*10^5 cells/ ml in 6-well plates. Each well was filled with 1.5 ml of cell suspension. The cells were infected the next day with either Timer-CVB3 or eGFP-CVB3 at an MOI of 100. Cell culture suspension was collected at 5 days post infection for isolating EMVs.

2.3.3 HeLa RW Cells

HeLa RW cells were cultured in complete media and seeded in 6-well plates or µ dishes as required by the experiment at a concentration of 1*10^5 cells/ ml. Three ml of media with cells was used per well for 6-well plates and 2 ml was used for µ dishes. Cells were incubated overnight at 37°C with 5% CO₂ and infected the next day with either CVB3 virus or infectious EMVs.

2.3.4 Wild Type and ATG5-KO Mouse Embryonic Fibroblasts

Wild Type (WT) and ATG5-KO mouse embryonic fibroblasts (MEFs) were cultured in complete media (1X DMEM, 10% FBS) with 1% Penicillin/Streptomycin (P/S) in gelatin fibronectin coated wells. Cells were seeded at a concentration of 1*10^5 cells/ml in gelatin/ fibronectin coated 6-well plates. After an overnight incubation at 37°C and 5% CO₂, cells were counted and infected with Timer-CVB3 with various MOIs and subsequently imaged at 8 hours, 24 hours and 48 hours post infection. At each time point, 200 µl of sample was collected and viral titers were measured by plaque assay.

2.3.5 Chinese Hamster Ovary (CHO) Cells

CHO cells were cultured in complete media and seeded overnight in 6 well plates or µ-dishes at a concentration of 1*10^5 cells/ ml. Separately, EMVs were isolated from eGFP-
CVB3 infected C2C12 cultures. CHO cells were then infected with EMVs and the infection was tracked by fluorescent microscopy.

2.4 ISOLATION OF EXTRACELLULAR MICROVESICLES

EMVs were isolated by two different techniques: by using a commercially available kit and by ultracentrifugation and is described below.

2.4.1 EMV Isolation by EXOQUICK-TC

Extracellular microvesicles (EMVs) were isolated from cell culture suspensions of differentiated neurospheres and C2C12 myoblast cells. Neurospheres were seeded at a concentration of 1*10^5 cells/ml in gelatin/ fibronectin coated micro dishes. They were differentiated for five days and the media was changed every two days. The cells were infected with eGFP-CVB3 (moi=0.1). On day 3 PI, EMVs were isolated using Exoquick-TC exosome isolation kit (System Biosciences, Inc, Cat# EXOTC10A-1). Cell supernatants were centrifuged at 3000 g for 15 minutes to pellet cell debris. Supernatants were transferred to a fresh conical tube. Exoquick-TC reagent was added at a 1:5 dilution (2 ml of Exoquick-TC to every 10 ml of cell culture supernatant). After an overnight incubation at 4°C, the sample was centrifuged at a 1500 g for 30 minutes. The EMVs separated as a pellet. Alternatively, C2C12 cells were seeded in 6-well dishes at a concentration of 1*10^5 cells/ml. The cells were infected the next day with eGFP-CVB3 (moi=100). On day 5 PI, EMVs were purified and separated as a pellet using the protocol described above for NPSC EMVs. The pellet was resuspended in 100µl of PBS. 20 µl of this suspension was placed on a slide and was visualized on a fluorescent microscope at a magnification of 320X. Also, HeLa cells were infected with 50 µl of EMVs and were imaged by fluorescent microscopy at various time points. Alternatively, purified EMVs were resuspended in 1X DMEM. The EMV and non-EMV fractions were freeze-thawed 3 times and the viral titers were determined by plaque assay.

2.4.2 EMV Isolation by Isopycnic Gradient Centrifugation

C2C12 myoblast cells were seeded at a concentration of 1*10^5 cells/ml in T-25 flasks containing 1X DMEM, 10% FBS and 1% antibiotics. The next day, cells were infected
with eGFP-CVB3 (moi of 100). An 8-20% iodixinol gradient was established (Opti-Prep, Sigma-Aldrich). One ml of the 5 days PI C2C12 cell culture supernatant was slowly added to the 8-20% gradient and the gradient was centrifuged at 141,000 g in a SW. 41 Ti rotor for 48 hours at 4°C in a Beckman L8-60 Ultracentrifuge. A total of 24 fractions were collected from the top of the tube and the viral titers were measured by plaque assay. The density of each fraction was determined by using a Bausch and Lomb Refractometer (Bausch and Lomb, Inc).

2.5 Western Blotting

NPSCs or C2C12 Myoblast cells were seeded and infected as described above. Scraped cells or purified EMVs were washed with PBS and lysed using cell extraction buffer. The suspension was chilled on ice for 30 minutes, vortexing every 10 minutes. The protein concentration was determined by a bicinchoninic acid assay and 20 µg of protein was utilized for protein analysis by western blotting. The primary antibodies used for western blotting were: rabbit anti-Flotillin-1 (Cell Signaling Technologies, Inc, Cat# 3253), rabbit anti-LC3 A/B (Cell Signaling Technologies, Inc, Cat# 4108), and mouse anti-enteroviral VP1 (Vector Laboratories Cat# VP-E603).

2.6 Neutralization Assay

C2C12 cells were seeded in 6 well plates at a concentration of 1*10^5 cells/ml in triplicates. After an overnight incubation, cells were infected with eGFP-CVB3 (moi=100). On day three p.i, EMVs were isolated as a pellet as described before using Exoquick-TC. EMV fraction was separated from the non-EMV fraction. Both fractions were serially diluted. All samples were then incubated in anti-CVB3 antibody (1:3000) (Millipore, Inc, Cat# MAB948) and rocked for two hours at 4°C. The viral titers of each sample were calculated by performing plaque assays.

2.7 Fluorescent Microscopy

All cells were imaged live at specified time points using the Ziess Inverted Fluorescent microscope. Three to five representative images were taken at each time point at magnifications of 200X or 320X.
2.8 Flow Cytometry

C2C12 cells were seeded in T-75 flasks at a concentration of 1*10^5 cells/ml. The next day, cells were infected with eGFP-CVB3 (moi=100). On day 5 pi, EMVs were isolated from supernatants as described before. EMVs were fixed by resuspending the EMV pellet in 200 µl of FACS buffer. EMVs were then stained using a PE-anti-LC3-II antibody (Cell signaling). Flow analysis was performed using the PE channel of BDFACS machine at the SDSU core facility.
CHAPTER 3

RESULTS

3.1 CHARACTERIZE EXTRACELLULAR MICROVESICLES SHED BY CVB3 INFECTED CELLS

Recently it was shown that a non-enveloped picornavirus, hepatitis A, acquires a host membrane from the ESCRT pathway and exits the infected cell encapsulated in this membrane [14]. This suggests that non-enveloped viruses may have developed mechanisms to escape into the extracellular environments undetected. Studies on poliovirus have revealed that the autophagy pathway may be involved in the release of virus from the cell [21]. Recently, our laboratory has shown that autophagy is up regulated in HL-1 cells infected with CVB3. Also, other researchers have shown that that CVB3 infection induces the formation of autophagosomes without undergoing degradation by lysosomes [22]. In contrast, a reduction in autophagy was observed in partially differentiated NPSCs infected with CVB3 [26]. Also, microvesicles comprising viral protein were observed when partially differentiated NPSCs or C2C12 myoblast progenitor cells were infected with Timer-CVB3 [11]. While some of these microvesicles remained intracellular, others were extracellular and seen in close proximity to infected cells. I wished to characterize these microvesicles and determine if the autophagy pathway was involved in their release.

3.1.1 Shed Microvesicles Were Observed in Partially Differentiated NPSCs Infected with Timer-CVB3

NPSCs were differentiated for five days in differentiation media. On day five, cells were infected with Timer-CVB3 (moi=0.1) and imaged at day 2, day 3 and day 4 p.i. Intracellular vesicles as well as some extracellular microvesicles lining the cell membrane were observed in infected cells. Some of these vesicles also expressed viral protein as seen in Figure 3.1. Some intracellular microvesicles expressing viral protein were observed (indicated by white arrows) and while these may represent replication organelles as described before [30], some extracellular microvesicles were also observed. In order to determine the source of these microvesicles further analysis by protein quantification was carried out.
Figure 3.1. Fluorescent microscopy of partially differentiated NPSCs infected with Timer-CVB3. Intracellular and Extracellular vesicles are indicated by white arrows. Cells were imaged at a magnification of 200X or 320X as indicated.

3.1.2 Purified EMVs Express Viral and Autophagosomal Proteins

Partially differentiated NPSCs were infected with dsRed-CVB3 and transduced with an adenovirus expressing GFP-LC3(Adeno-GFP-LC3). By day 3 PI, abundant EMVs expressing both viral protein and GFP-LC3 were observed (Figure 3.2A-3.2F). These results suggested that the autophagy pathway was involved in the microvesicle egress from the cell following CVB3 infection. To further characterize these microvesicles, partially differentiated NPSCs were infected with Timer-CVB3 (moi=0.1) and microvesicle release was tracked using fluorescent microscopy. On day 3 PI, microvesicles were precipitated from supernatants using Exoquick-TC™. Western blots were carried using primary antibodies for viral capsid protein 1(VP1), exosomal protein-Flotillin-1 and autophagosomal protein-LC3. Shed microvesicles expressed all these markers as shown in Figure 3.2 (G). The complete detection of LC3-II protein in western blots suggest that EMVs are mature, double-membrane autophagosomes and possibly exit the cell as single membrane vesicles by fusing with the plasma membrane [31].
3.1.3 High Levels of Infectious Virus Associated with EMVs

EMVs were isolated from partially differentiated NPSCs and C2C12 myoblast cells following infection with CVB3 using *Exoquick-TC* isolation. The EMV pellet was diluted in equal volumes of 1X DMEM as the supernatant fraction. A standard plaque assay was carried out to titer the amount of infectious virus associated with each sample. EMV precipitate fractions for both the NPSCs and C2C12 samples comprised a higher concentration of virus compared to the supernatant fraction (Figure 3.3 A-C). This suggests a preferential association of virus with shed EMVs released by progenitor cells. Freeze-thaw treatment of EMVs showed considerable reduction of viral titers. In contrast, freeze-thaw treatment of the supernatant fraction showed no reduction in viral titers (Figure 3.3 D).
3.2 High Numbers of LC3-II⁺ EMVs Released by Infected Cells Were Detected by Flow Cytometry

In order to determine the quantity of EMVs released by infected cells over time and to better characterize EMV surface markers, these microvesicles were analyzed by flow cytometry. C2C12 cells were infected with either eGFP-CVB3 or mock infected and EMVs were harvested as described before. EMVs were stained with a PE conjugated antibody specific for LC3-II and analyzed by flow cytometry. A large number of LC3-II⁺EMVs were observed in CVB3-infected samples as compared to mock infected cells (Figure 3.4). Also, the total number of LC3-II⁺ EMVs produced by infected cells was higher than the total number produced by mock-infected cells confirming that CVB3 infection induced the formation of EMVs most likely derived from the autophagy pathway.

3.3 Assess If EMVs Can Establish New Infection

Recently, Hepatitis A virus (HAV), another picornavirus has been shown to hijack the host ECSRT pathway to release virions encapsulated in a host-derived membrane. The enveloped form of HAV was the dominant form of virus released form infected cells and was shown to be infectious [14]. This new mechanism of viral egress from infected cells changes the way we perceive picornavirus infections. EMVs released from infected cells may represent a novel mechanism of cell-cell transfer of infectious virus. The following
Figure 3.4. FACS of EMVs isolated from infected and control C2C12 cultures. High numbers of LC3-II+ EMVs were released by infected cells as compared to mock infected cells. Also, the total microvesicles produced by CVB3 infected cells were higher than mock infected cells.

experiments were designed to determine if EMVs released by CVB3 infected cells were infectious and could initiate a new infection in HeLa cells.

3.3.1 Purified EMVs Were Infectious

As shown in Figure 3.5 B, purified EMVs were successful in infecting HeLa cells. EMVs were isolated from infected C2C12 supernatants using the Exoquick-TC precipitation technique. The pellet was then resuspended in 1X PBS and inspected for viral protein (eGFP) using fluorescence microscopy. The diameter of microvesicles was calculated using the Length Interactive Measurement feature of the Axiovision software. Microvesicles expressing high levels of viral protein varied widely in size, ranging between 0.51 µm-5.53 µm in diameter (Figure 3.5 C). eGFP+ EMVs were then tested for infectivity by incubation with HeLa cell cultures. eGFP+ EMVs added to HeLa cells were observed by fluorescent microscopy over time (0 and 1 hr PI) as seen in Figure 3.4 B. The eGFP+ EMVs were no longer visible by 3 hours PI, and may have undergone a fusion event. Viral protein expression and cytopathic effects were seen in HeLa cells by 24 hours post infection.
Figure 3.5. Purified EMVs are infectious. (A) Purified eGFP+-EMVs isolated from infected C2C12 cells, observed by fluorescent microscopy at a magnification of 320X. (B) Representative images of HeLa cells infected with eGFP+-EMVs at specified time points. (C) Size distribution of isolated EMVs calculated using the Axiovision Length Interactive Measurement Software.
suggesting that EMV mediated infection in HeLa cells mimics infection carried out by non-enveloped virions.

### 3.3.2 Infectious EMVs Were Found in Low Density Isopycnic Iodixanol Gradients

In order to show that virus encapsulated virions were distinct from free virions, I purified EMVs using Isopycnic gradient centrifugation. Supernatants from C2C12 cells, infected with eGFP-CVB3 (moi=100), were run through 8-20% iodixanol gradient to resolve 24 fractions (Figure 3.6). A high level of infectious virus was observed in fractions 22-24 which reflect the expected density of picornavirus virions (1.22 g/cm$^3$).

![Figure 3.6](image)

Figure 3.6. Ultracentrifugation of infected C2C12 supernatants. Buoyant density of infectious encapsulated virus was in the range of 1.04-1.10 gm/cm$^3$. Non-enveloped infectious virus had a density of 1.22 gm/cm$^3$.

A comparable level of infectious virus also associated with fractions 6-21, which fall in the range of membrane associated virions (1.04-1.1 g/cm$^3$). While exosome associated hepatitis A enveloped virions were reported to have a small density range of 1.06-1.10 g/cm$^3$ [14], CVB3-associated EMVs showed a broader density range.
3.3.3 Determine if EMVs Expand Tropism

Infection of host cells by a virus involves four major steps: Interaction and binding of virus with cell surface receptors, uptake of virus, virus replication, and exit from the host cell. The Coxsackie and Adenovirus receptor (CAR) mediates the uptake of CVB3 virions into the host cell. The presence of this receptor is crucial for infection and cell-cell viral dissemination. While free virions require this surface receptor for viral entry, encapsulated virus may not need it. I wished to test if EMVs could bypass receptor-mediated virus entry and initiate infection in CAR\(^-\) cells. Chinese Hamster Ovary (CHO) cells are non-permissive toward the entry of CVB3 as they lack CAR. CHO cells inhibit viral entry but support infection when transfected with CVB3 cDNA or viral RNA, suggesting that once receptor-mediated entry is bypassed, these cells might produce infectious virus [32]. Therefore, EMVs were isolated from eGFP-CVB3 infected C2C12 cells or dsRED CVB3 infected C2C12 cells as described before. Separately, HeLa cells were used as control in each experiment to account for infectivity of EMVs. Both eGFP-CVB3\(^+\)-EMVs and dsRED-CVB3\(^+\) were unsuccessful in infecting CHO cells, but were able to infect HeLa cells as shown in Figure 3.7. This implies that EMVs also require CAR receptor for entry into host cells. Since EMV isolation requires centrifugation and an additional 24-hour incubation, the EMV membranes may have disrupted in the procedure. This could be the reason CHO cells were not susceptible to infection as the virus may no longer be encapsulated but rather released from membranes as free virions, which would require receptor mediated entry. It could also be possible that the CHO cells used for this experiment cannot support CVB3 infection even if transfected with viral RNA. Therefore a control experiment, showing that CHO cells can be transfected with CVB3 RNA needs to be carried out.

3.4 Examine if Infectious EMVs Evade the Host Immune System

The mechanism of cell to cell dissemination for CVB3 is still unclear and under investigation. The formation of infectious EMVs post infection in progenitor cells undergoing differentiation suggests that CVB3 has adopted a novel mechanism to escape host cells. I wished to determine if infectious EMVs are invulnerable to host neutralizing antibodies. Encapsulation of virus by autophagosome or exosome associated membranes
would protect the virus from host neutralization. Perhaps the virus has evolved to harness this mechanism to persist in the host.

### 3.4.1 EMVs Were Susceptible to Neutralization by Anti-CVB3 Antibody

C2C12 cells were plated and infected with eGFP-CVB3 (moi=100) in triplicate cultures. On day three post infection, EMVs were harvested using the *Exoquick-TC* protocol. EMV pellet fractions were separated from the non-EMV fractions and each sample was serially diluted with dilution factor of $10^{-1}$. To neutralize the virus, all samples were incubated with an anti-CVB antibody (dilution of 1:3000). After incubating for two hours, plaque assays were carried out to determine the viral titers post neutralization. Separately, standard plaque assays were carried out to determine the titers associated with each undiluted sample. The data included in Figure 3.8 represents $10^{-3}$ dilution. There was a significant reduction in viral titers and the number of plaques of EMVs incubated with the neutralizing antibody as compared to the untreated EMVs (Figure 3.8A-3.8 B). These results suggest that the EMVs were neutralized by the addition of CVB3-specific antibodies and encapsulation was unable to protect the encapsulated virus. Another possibility could be that the EMVs are unstable and became disrupted during isolation (as explained previously). A more delicate
EMVs are susceptible to neutralization. (A) Number of plaques formed in EMV and non-EMV fraction, in presence of antibodies and no treatment condition (dilution of $10^{-3}$). (B) Viral titers of EMV and non-EMV fractions, in presence of antibodies and no treatment condition (dilution of $10^{-3}$). (C) Viral titers of original samples before diluting them.

protocol to isolate EMVs might need to be devised. If the EMVs are no longer intact and virus is no longer encapsulated – this consequence may account for the reduction of viral titers in presence of antibodies.

### 3.4.2 Determine the Role of Autophagy in Dissemination of CVB3

In order to determine if the EMVs are released from the autophagic pathway, I infected ATG5 knock out (ATG5-KO) and wild type (WT) mouse embryonic fibroblasts (MEF). ATG5-KO MEFs were isolated from mice defective in the ATG5 gene. The ATG5 gene is crucial for the formation of autophagosomes. During the earlier steps of autophagy, ATG5 pairs with ATG7 to recruit LC3II in order to form autophagosomes. In absence of this gene, the cell is impaired in its ability to form autophagosomes. I hypothesized that cell to cell infectivity of CVB3 would be greatly reduced in ATG5KO MEFs since these cells would lack virus-encapsulating autophagosomes. The results of my previous experiment suggested that EMVs may be getting disrupted in the isolation procedure and as a result were susceptible to neutralization. In order to avoid this, I infected WT and ATG5KO-MEFs with Timer-CVB3 for one hour and then washed the cells. This would facilitate the entry of virus in the cells. After an hour of infection, the cells were washed and a neutralizing anti-CVB3 antibody was added at a dilution of 1:3000. Addition of the neutralizing antibody would restrict the dissemination of free virions. Cells were imaged at 8 hr, 24 hr and 48 hr p.i and supernatants were collected at each time point to measure viral titers (Figure 3.9).
Figure 3.9. Role of autophagy in the dissemination of CVB3. (A) Representative images of WT and ATG5KO-MEFs infected with Timer-CVB3 (MOI=1) in presence and absence of neutralizing antibody. (B) Representative images of WT and ATG5KO-MEFs infected with Timer-CVB3 (MOI=10) in presence and absence of Neutralizing antibody. (C) Quantification of triplicate samples of WT and ATG5KO-MEFs infected with Timer-CVB3 (MOI=1or 10) in presence and absence of neutralizing antibody.

As seen in Figure 3.9, addition of the neutralizing antibody reduced titers of both WT and ATG5KO cell supernatants. This suggests that the EMVs may be susceptible to neutralization. Also, the higher amount of infectious virus present in the ATG5KO-MEF supernatants as compared to the WT-MEFs suggests that, additional mechanisms of releasing viral progeny may be involved during infection. I suspect that the initial high viral titers in ATG5KO-MEF supernatants at 8h PI could be because these cells have a tendency to grow
faster than the WT-MEFs and, therefore, received a higher amount of virus to account for the extra cells and appropriate MOI. This may be one of the reasons why the ATG5KO supernatants maintained high viral titers throughout the infection since these cells received a higher amount of virus inoculum than the WT-MEFs. Also, one hour of initial infection may not be sufficient for the virus to enter WT-MEFs. This experiment will be repeated with a four hour initial infection period to ensure that the virus has sufficient time to enter cells. Also, neutralizing antibody will be added after every 24 hours to ensure that non-encapsulated virions are neutralized as they are released from the cells.
CHAPTER 4

DISCUSSION

Enteroviruses are significant human pathogens that can cause long term neurological diseases. Determining cell to cell dissemination of virus is important in order to understand viral pathogenesis and persistence in the host. Several viruses use host mechanisms in order to promote their replication and proliferate in the host [23, 25]. The mechanism of cell to cell spread of CVB3 and persistence in the host is still not fully understood. In vivo, CVB3 first infects the pancreas, and after replication, the virus may disseminate to other target organs such as the heart and the CNS. We recently developed a recombinant CVB3 expressing “fluorescent timer: protein” (Timer-CVB3) that allowed us to track viral pathogenesis in real time. “Fluorescent timer” protein acts as a marker for infected cells that progressively changes color from green to red. Upon infection with Timer-CVB3, NPSCs and C2C12 myoblast cells appeared to release a large numbers of extracellular microvesicles (EMVs). My research was focused on characterizing these EMVs, determining if EMVs play a role in cell to cell viral dissemination, assessing if these EMVs are generated from the autophagy pathway. Also, I wished to inspect whether EMVs are immune to host neutralizing antibodies.

The release of microvesicles from the host cell is important for cellular communication and to regulate immune responses. Cells release highly specific microvesicles depending upon the cell cycle stage or the stage of infection [13, 32]. These microvesicles could benefit the virus by providing protection from the immune system [14]. Alternatively, microvesicles could benefit the host by serving as antigen markers to stimulate dendritic cells [23]. While the release of microvesicles by infected cells have been extensively studied in enveloped viruses such as HIV, herpes simplex virus and Epstein-Barr virus, the field remains unexplored for coxsackieviruses. Recently, it was shown that HAV escapes immune recognition by hiding in host-derived membranes derived from hijacking the ESCRT pathway [14]. These results suggest that non-enveloped viruses may have evolved to behave more like enveloped viruses. Therefore, we need to further explore the mechanisms
of viral egress from the host cell and persistence for viruses historically considered as “non-enveloped”. Poliovirus (PV), another picornavirus, has been shown to induce the formation of double-membraned autophagosomes filled with viral particles [21, 33]. We have shown that Timer-CVB3 infection of NPSCs and C2C12 myoblast progenitor cells triggered the release of EMVs in culture [11]. These microvesicles contained mature timer protein and viral material, suggesting that these unique structures were not cellular debris. We hypothesized that CVB3 may be utilizing host-derived membranes in order to disseminate and persist in the host.

When partially differentiated NPSCs were transduced with LC3-GFP (using an adenovirus vector) and simultaneously infected with dsRED-CVB3, EMVs expressing both GFP-LC3 and virus protein (dsREd) were observed (Figure 3.2). This indicated that EMVs were associated with the autophagy pathway and harbored viral material. Western blot analysis of EMV pellet was positive for lipid raft marker-flotillin-1, mature autophagosome marker-LC3-II and viral protein-VP1 (Figure 3.2). The presence of LC3-II implicated that the EMVs represented mature autophagosomes which most likely failed to fuse with the lysosome and were ejected from cells. Previous studies have shown that autophagosomes support CVB3 replication [22]. Comparing the levels of virus associated with EMV and non-EMV fraction revealed that, a high titer of virus was associated with the EMV fraction. These data suggests that CVB3 preferentially associated with the EMV fraction as compared to the supernatant fraction (Figure 3.3). Previously, encapsulated HAV (eHAVs), released by HAV infection were shown to be sensitive to chloroform extraction [14]. EMVs were also sensitive to freeze-thaw treatment, and drastic reduction in viral titers was observed following disruption of these structures (Figure 3.3 D) as compared to the no treatment control. Flow cytometric analysis of infected C2C12 supernatants confirmed the presence of LC3-II+ EMVs, and also showed a higher number of vesicles released from infected as compared to mock infected cells (Figure 3.4). Many viruses have been known to subvert autophagy in order to enhance viral replication. In some cases viruses use this machinery to enhance replication. For example, rotavirus, another RNA virus hijacks autophagy membranes to transport viral proteins to sites of virus replication for assembly of infectious virions [25]. HAV, another picornavirus, has been shown to hijack the host ESCRT pathway to release virions wrapped in exosome-like membranes [14]. CVB3-associated EMVs may be
released by the autophagic pathway, similar to a process previously described
autophagosome exit without lysis (AWOL) for poliovirus [21]. Recently, our lab showed that
partially differentiated NPSCs revealed a lower amount of LC3\(^+\) autophagosomes following
infection [26]. These data contrasted with results utilizing a cardiac cell line (HL-1), and
contrasted with results by other investigators using various cancer cell lines [34, 35]. This
information, along with our recent EMV data suggests that these double-membrane
autophagosomes exit the cell as single-membrane EMVs, most likely by fusing with the
plasma membrane. Since we observed no change in the levels of LC3 puncta in
undifferentiated NPSCs infected with CVB3 [26], the differentiation process may be acting
as a trigger for the extracellular release of infectious EMVs.

I wished to determine if the isolated EMVs were capable of initiating a new infection
in HeLa cells. EMVs were successful in infecting HeLa cells, and by 24 hr PI, the dynamics
of infection were similar to those of CVB3 virions (Figure 3.5). Also, EMVs were separated
by gradient ultracentrifugation to determine if infectious virus was observed at a density
differing from that of loose virus, perhaps representing membrane-associated virus through
encapsulated EMVs. A broader range of densities for membrane-associated virus were found
for infectious EMVs as compared to infectious eHAVs (Figure 3.6). These results might
reflect the broad range of EMV sizes observed by fluorescent microscopy (Figure 3.5 C).

CHO cells lack the receptor for virus entry and thus cannot be infected by free CVB3
virions . I hypothesized that EMVs might expand virus tropism to non-CAR expressing cells
by fusing with plasma membrane and bypassing the need for receptor-mediated entry.
However virus-associated EMVs were not successful in infecting CHO cells (Figure 3.7).
Nevertheless, EMVs may be more delicate than we originally expected, and hence future
experiments will determine if virus-associated EMVs where disrupted and did not survive the
extensive isolation protocol. Encapsulated CVB3 virus may represent “immature” virions,
and therefore may be intimately associated with the membrane so that disruption of EMVs
may affect infectivity. Also, if the EMV membranes are no longer intact, these virions
released would require CAR for entry into target cells. Alternatively, CVB3 may not be able
to replicate upon entry into CHO cells due to host antiviral responses. In order to verify their
susceptibility to infection, CVB3 RNA will be transfected in CHO cells to determine if these
cells support CVB3 replication. Gentler isolation techniques may also be needed in order to keep the EMVs intact. Alternatively, another cell line lacking CAR could be utilized.

Finally, I wished to determine whether EMVs are resistant to neutralization by the host immune response. We determined that EMVs isolated from infected C2C12 myoblast supernatants were susceptible to neutralization by anti-CVB3 antibody [Figure 3.8 A, B]. There was a distinct reduction in the number of plaques formed and a reduction in viral titers for EMV fractions incubated with the antibody. However as mentioned above, EMV membranes may be sensitive to the extraction, and hence a different technique should be used to verify virus-associated EMV susceptibility to neutralization. The experiment could be improved by isolating EMVs by gradient ultracentrifugation, which will help test the broad range of membrane-associated virus fractions. In an effort to keep the EMVs intact and test if they were resistant to neutralization by host antibodies, ATG5-KO and WT MEFs were infected with Timer-CVB3. This experiment was also aimed at determining the role autophagy (ATG5 gene) in the shedding of virus-associated EMVs. By 8 hr post infection, viral titers and viral protein expression were drastically reduced in both ATG5-KO and WT cells incubated with the neutralizing antibody. These results show that, either the 1 hour infection period was not sufficient for the formation of EMVs and their release outside the cell or, EMVs maybe formed and once shed, they were sensitive to neutralization by the antibodies in the supernatants. Contrary to my expectation, the ATG5-KO cells sustained a higher amount of viral titers than the WT-MEFs. Previously, autophagy has been shown to be advantageous for replication and the dissemination of different viruses. I expected that since the ATG5KO cells lack the ability to form autophagosomes, they would not possess this additional pathway to promote virus dissemination, and hence these cells would support lower viral titers in their supernatants. There were also a few technical difficulties in the experiment which could explain the unexpected results. The ATG5KO-MEFs grew faster than the WT-MEFs and infecting ATG5KO MEFs at the optimal MOI required higher amount of virus to account for the additional cells. This could explain the higher amount of virus in the ATG5-KO supernatants at 8 hr post infection (Figure 3.9 C).

In summary, my research indicates that CVB3 infection triggers the release of virus-associated EMVs from infected NPSCs and C2C12 cells. These EMVs harbor infectious virus and can initiate new infection in host cells. Co-localization of VP1 and LC3 indicates
that these vesicles are autophagosomes which encapsulate virus for egress from the host cell. The presence of autophagy proteins in the EMV fractions indicate that these microvesicles may be derived from the autophagy pathway.

Future experiments will be focused on characterizing the formation of EMVs *in-vivo and* isolating EMVs from infected mice. Deciphering the pathway involved in the shedding of these EMVs may be crucial to designing new therapeutic targets against enteroviral infections. Use of autophagy inhibitors and/or spingomyelinase inhibitors will be used in the future to determine the role of autophagy in the formation of EMVs.
REFERENCES


