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Proceedings of the 2017 ISEV symposium on “HIV, NeuroHIV, drug abuse, & EVs”

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Abstract

Despite the success of combination antiretroviral therapy (cART), there is increased prevalence of HIV-associated neurocognitive disorders (HAND) in HIV-1-infected individuals on cART, which poses a major health care challenge. Adding further complexity to this long-term antiretroviral use is the comorbidity with drugs of abuse such as morphine, cocaine, and methamphetamine, which can in turn, exacerbate neurologic and cognitive deficits associated with HAND. Furthermore, HIV proteins, such as the transactivator of transcription (Tat) and the envelope protein (gp120), as well as antiretrovirals themselves can also contribute to the progression of neurodegeneration underlying HAND. In the field of NeuroHIV and drug addiction, EVs hold the potential to serve as biomarkers of cognitive dysfunction, targets of therapy, and as vehicles for therapeutic delivery of agents that can ameliorate disease pathogenesis. Based on the success of a previous Satellite Symposium in 2015 at the ISEV meeting in Washington, experts again expanded on their latest research findings in the field, shedding light on the emerging trends in the field of Extracellular Vesicle (EV) biology in NeuroHIV and drug abuse. The satellite symposium sought to align experts in the fields of NeuroHIV and drug abuse to share their latest insights on the role of EVs in

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Compliance with ethical standards

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regulating neuroinflammation, neurodegeneration, peripheral immune response, and HIV latency in HIV-infected individuals with or without the comorbidity of drug abuse.

Keywords

Extracellular vesicles (EVs); Human immunodeficiency virus (HIV); HIV-associated neurocognitive disorders (HAND); NeuroHIV; Drug abuse

Introduction

In conjunction with the annual meeting of the International Society for Extracellular Vesicles (ISEV), a satellite symposium on “HIV, NeuroHIV, drug abuse & EVs” was held on May 17th, 2017 in Toronto, Canada. The workshop was an extension of a satellite symposium held at the 2015 I.E. meeting in Washington (Hu et al. 2017) and was organized by Dr. Shilpa Buch from the University of Nebraska Medical Center and Dr. Kenneth Witwer from Johns Hopkins University School of Medicine with the support of Dr. Andrew Hill, from La Trobe University and the ISEV organizing committee. Dr. Hill as the President of ISEV2017 provided the introductory remarks by sharing his enthusiasm about this symposium and commenced the session, which was chaired by Drs. Fatah Kashanchi and Shilpa Buch. The satellite symposium was open to all the ISEV attendees.

Extracellular vesicles (EVs) comprising of microvesicles, exosomes, and apoptotic bodies act as cargo-carrying vesicles mediating communication among diverse cell types and tissues, including the central nervous system (CNS). Recently, EVs have gained much attention for their involvement in the regulation of important biological processes such as immune responses and cell differentiation and their potential to contribute to disease etiology. Studies have implicated the role of EVs in a variety of diseases including the progression of neurological disorders and cancers. The differential release of EVs, varying cargo and subsequent biological functions, and potential therapeutic applications of EVs in HIV-associated neurocognitive disorders (HAND) and drug abuse have been extensively investigated, widely reported, and vigorously discussed—all yielding significant progress over the last few years.

Drugs of abuse, such as opiates, cocaine, and methamphetamine, potentiate neuroinflammation, and neurodegeneration are associated with HIV-1 infection. Recent studies have demonstrated that HIV proteins (Gag and Nef) and RNA (the trans-activation response element (TAR) miRNA) are secreted into EVs (Booth et al. 2006; Campbell et al. 2008; Narayanan et al. 2013; Rahimian and He 2016; Sami Saribas et al. 2017; Shelton et al. 2012) and that these viral components can be transferred to neighboring/distant cells leading to functional impairment. In an elegant study, Narayanan and colleagues have demonstrated that EVs released from HIV-1-infected cells contain both 5' and 3' TAR miRNAs, as well as the viral proteins, such as Tat, Gag, and Env (Narayanan et al. 2013; Rahimian and He 2016). The presence of TAR RNA was further confirmed in serum EVs isolated from HIV-1-infected patients (Narayanan et al. 2013). Intriguingly, prior exposure of uninfected cells with EVs isolated from infected cells makes the recipient cells more susceptible to HIV-1 infection and confer a protective phenotype to these recipient cells under conditions of

cellular stress (Narayanan et al. 2013). Additionally, HIV-infected cells release HIV proteins, such as Tat and Nef, in EVs, which, following uptake by noninfected bystander cells leads to the release of proinflammatory cytokines and/or apoptosis (Lenassi et al. 2010; Rahimian and He 2016; Sampey et al. 2016). In addition to viral components, host miRNAs, proteins, and their expression in EVs released from viral infected and/or drug exposed cells have also been well characterized (Hu et al. 2012; Konadu et al. 2015b; Roth et al. 2015; Tang et al. 2016). As an example, the level of miR-29b in EVs released from morphine and HIV Tat-exposed astrocytes is significantly increased. These EVs can be taken up by neurons resulting in a concomitant decrease in the expression of PDGF-B, an important target of miR-29, and reduced viability in these latter cells (Hu et al. 2012). Interestingly, Yelamanchili and colleagues have demonstrated for the first time that brain-derived EVs show significant upregulation of miR-21 in SIV encephalitis. Furthermore, these authors showed that EV-miR-21 could activate the TLR7-dependent downstream necroptosis pathway, thus leading to neurotoxicity in a model of SIV neuropathogenesis (Yelamanchili et al. 2015). A subsequent study by the same group showed upregulation of EV-miR-21 in traumatic brain injury (TBI) (Harrison et al. 2016).

Emerging evidence also suggests that plasma EVs serve as important mediators of periphery-to-brain communication. The quantity of EVs and associated cargo (such as miRs and proteins) found in the plasma of HIV+ drug abusers was found to be significantly altered compared with the corresponding controls. The underlying mechanism(s) by which plasma EVs affect the brain and, in turn, contribute to the progression of HIV-1 and/or manifest symptoms of drug abuse, however, have not been well understood. Studies by Dickens et al. have demonstrated that EVs shed from astrocytes in response to interleukin-1 β (IL-1 β) can rapidly cross the blood-brain barrier (BBB) and enter the peripheral circulation and the liver, leading to regulation of expression of inflammatory cytokines via suppression of PPAR α , finally promoting the transmigration of leukocytes into the CNS (Dickens et al. 2017). This study underscores the role of astrocytic EVs as important mediators of communication between the brain and peripheral immune system and as pivotal regulators of immune response within the CNS (Dickens et al. 2017).

More recently, exciting studies have also highlighted alterations in plasma neuron-derived EVs (NDEs) during HIV infection (Sun et al. 2017). Intriguingly, in this study, the authors demonstrated that NDEs were influenced by both HIV infection as well as neurocognitive impairment, as evidenced by increased expression of stress proteins such as HMGB1, neurofilament-1, and amyloid beta protein in the NDEs compared with plasma exosomes. This study underscores the utility of NDE protein profiling as new liquid biopsy biomarkers for cognitive impairment.

Collectively, presentations by experts in the field of NeuroHIV and drug abuse reported substantial accomplishments in the EV field ranging from improved isolation methods, characterization, composition, functions, and application of EVs (DeMarino et al. 2017; Dickens et al. 2017; Gardiner et al. 2016; Hu et al. 2016; Konadu et al. 2016; Sampey et al. 2014; Tang et al. 2016; Witwer et al. 2013; Yelamanchili et al. 2015). The satellite symposium brought together leaders in the field to share their most recent findings and insights.

Dr. Andrew F. Hill welcomed the attendees and expressed his enthusiasm and support for this symposium.

Dr. Shilpa Buch gave a talk entitled “Tat and Opiates modulate HAND: Role of astrocyte derived exosomes.” She gave a brief background on opiates such as morphine and described their pervasive clinical use (and abuse) based on their potency and efficacy as gold standard regimens for pain management. Both in vitro and in vivo studies have demonstrated that morphine potentiates the neurodegenerative effects of HIV in the CNS. Altered microglial functions such as attenuated phagocytosis and improper immune status have been implicated in mediating neurodegeneration underlying many CNS diseases. In recent times, the role of long intergenic noncoding RNAs (lincRNAs) in the regulation of cellular processes is gaining attention. While lincRNAs are known to maintain cellular homeostasis, dysregulation of their expression has been implicated in altered expression of a wide array of genes including those controlling phagocytosis. Based on this, it was hypothesized that EVs released from morphine-exposed astrocytes are taken up by microglial cells leading in turn, to impaired microglial phagocytosis via induction of lincRNA-Cox2 through the TLR-NF- κ B axis. In this study, mouse primary astrocytes and human A172 astrocytoma cells were exposed to morphine (10 μ M) followed by isolation of EVs using standard differential ultracentrifugation and their characterization using transmission electron microscopy, NanoSight, and western blot analyses. Expression of lincRNA-Cox2 in EV-treated BV2 cells and mouse primary microglial cells was examined by qPCR, and microglial phagocytosis was assessed by uptake of fluorescently labeled latex beads. In vivo studies involved intranasal delivery of lincRNA-Cox2 siRNA to mice that were administered morphine. Interestingly, EVs released from morphine-exposed astrocytes demonstrated upregulation of miR-138, which in turn, was shown to bind to endosomal TLR7 in microglia, leading to activation of the NF- κ B pathway. This, in turn, resulted in upregulation of lincRNA-Cox2, leading to impaired microglial phagocytosis. Intranasal delivery of lincRNA-Cox2 siRNA restored microglial phagocytic activity in morphine-treated mice. Taken together, it was shown that exposure of microglial cells to EVs released from morphine-exposed astrocytes resulted in impaired phagocytic function via the TLR7-NF- κ B-lincRNA-Cox2 axis. These findings have ramifications for the development of EV-loaded RNA drug target(s) as therapeutics for neurodegenerative disorders where restoration of altered gene expression may be beneficial.

Dr. Fatah Kashanchi presented his data on the “Effect of anti-retroviral drugs on exosome production.” HIV-1 is a complex retrovirus that produces chronic infection affecting approximately 1.1 million people in the USA with an estimated 50,000 new cases each year, yet there is still no known cure for this disease. To date, the most effective treatment for HIV is a combination of several antiretroviral drugs (cART), which lowers viral titers and reverses pathology. Dr. Kashanchi’s group investigated the effect of cART on the quantity and content of exosomes released from HIV-infected cells. Previously, his group had reported that HIV-infected exosomes contain a non-coding RNA, trans-activating response (TAR) element RNA, which has been shown to increase susceptibility to HIV infection. Most recently, they also found enhanced exosome release when infected cells were treated with cART. In addition, there was altered expression of full length genomic RNA while other RNAs, such as TAR RNA, remained unaffected by the addition of cART treatment in

both cell lines and primary macrophages. These results agree with previous literature revealing that several other FDA-approved drugs have the ability to alter the number and content of exosomes released from HIV-infected cells. To further explore the variation in exosome quantity in response to cART treatment, the ESCRT pathway was assessed for changes in protein expression. Several ESCRT proteins were differentially expressed in cART treated cells, implying that antiretrovirals have off target effects on cellular proteins that are necessary for the release of virus or exosomes.

Dr. Lynn Pulliam presented a new concept for biomarker research entitled “Plasma neural-derived exosomes as biomarkers of cognitive impairment in HIV.” Looking at peripheral activation markers on monocytes has been a fairly accurate measure of comorbidity complications in HIV infection; however, monitoring damage to the brain in real time has been a real challenge. Finding an inexpensive, noninvasive, peripheral biomarker for cognitive impairment has been a high priority. Exosomes are shed from most cells including neural cells. Neuron-derived exosomes (NDE) were isolated from plasma of control and HIV-infected subjects with varying degrees of cognitive impairment. They examined these NDE for markers of neuronal damage. Total exos were isolated from plasma of 12 healthy control subjects and 23 HIV-infected subjects using Exoquick. All HIV-positive subjects were on antiretroviral therapy with none to varying degrees of cognitive impairment; most had controlled viral load. Further isolation to NDE was performed by immunoadsorption using antibody to the neuron specific cell surface marker L1CAM. NDE were characterized by neurofilament light (NF-L) and synaptophysin (SYP). They quantified exosome numbers, CD81, CD63, and protein content by ELISA for two targets associated with neurodegeneration, amyloid beta ($A\beta$) and high-mobility group protein B1 (HMGB1). Results showed that HIV-infected subjects had significantly fewer total plasma exosomes compared to controls but there was no difference in NDE; NF-L and SYP were elevated in NDE. Neuropsychologically impaired (NPI) subjects had significantly fewer NDE. HMGB1, $A\beta$, and NF-L were increased in NDE from impaired compared to normal subjects. In conclusion, they demonstrated for the first time that circulating neuron-derived exosomes show damage that correlates with cognitive impairment. The number of total exos in the plasma of individuals with HIV infection was decreased. Subjects with cognitive impairment had decreased NDE suggesting fewer neurons. However, in NDE, there was a significant increase in several targets including the alarmin, HMGB1 from impaired subjects. NDE cargo reflected neuronal damage and may predict a cognitive impairment profile (Sun et al. 2017).

Dr. Norman Haughey gave a talk on, “Deconstruction of Neural Networks by HIV-Associated Modifications in Exosome Cargo.” Astrocyte to neuron communication regulates the formation and strength of synaptic connections. However, relatively few mediators of this gliotransmission have been identified. Astrocyte-derived extracellular vesicles (ADEV) are emerging as a mechanism by which astroglia regulates synaptic connectivity through the delivery of protein, RNA, and lipid cargo. In this study, they isolated ADEV shed in response to ATP (a trophic stimulus), $TNF\alpha$, or $IL-1\beta$ (inflammatory stimuli) and exposed primary rodent neurons to a dose response of 5–100 ADEV/cell. The effects of EVs on dendritic length, branching, complexity, and spine maturation were determined using structured fluorescent imaging; and network activity was determined using multichannel

electrode arrays. They found that ADEV shed in response to ATP produced a dose-dependent increase in dendritic complexity, enhanced dendritic spine maturation, increased neural network connectivity, and promoted synchronous network burst activity. To identify mechanisms for this effect they first conducted whole genome sequencing of neurons exposed to ADEVs shed in response to ATP stimulation and identified 546 transcripts with expression changes ± 2 SD (192 increased and 354 decreased). A number of these transcriptional products regulate synaptic strength, receptor trafficking, or expressed scaffolding proteins that coordinate the localization and function of receptors to pre- and post-synaptic specifications. Interrogation of the proteomic and miRNA cargo of ADEVs shed in response to ATP and alignment of this molecular cargo with changes in gene expression of the target neurons identified integrin $\beta 1$, and ephrin receptors as regulators of ADEV enhanced neural network connectivity. We confirmed that integrin $\beta 1$ was required for binding of ADEVs to the neuronal surface and that ephrin signaling was involved in the downstream effects of ADEVs on neural network activity through pharmacological and molecular modulation of ephrin signaling. In contrast to this trophic response, ADEV shed in response to TNF α or IL-1 β deconstructed neural networks through the actions of specific miRNA. These findings demonstrate that stimulus-dependent modification of ADEV cargo can regulate a neurotrophic or neurodegenerative responses (Dickens et al. 2017).

Dr. Vincent C. Bond talked on “Hallmarks of HIV Pathogenesis are Modulated by Nef Secretion Modification Region.” His group has developed a model describing the role of Nef-exosomes (exNef) in HIV, hypothesizing that CD4 depletion and immune activation are driven via effects of exNef. To test this hypothesis, mutant viruses were developed with changes in the secretion modification region (SMR) of Nef, identified in earlier research (Ali et al. 2010; Konadu et al. 2015a). These mutations should affect exNef composition and secretion as reflected in cell culture assays and in an in vivo infection model. In cell culture, (1) no differential growth of mutant versus wild type (wt) virus during multi-round analysis was seen, while significant differences in single-round growth were observed. Thus, effects on pathogenesis were not due to viral growth differences, and (2) differential effects on CD4 T-cell depletion in 12-day infections relative to CD3 cells were seen, while differential apoptosis induction by exosomes of mutant versus wt virus-infected PBMC's supported the CD4 depletion result. Next, humanized (Hu)-mice (Konadu et al. 2015a) were infected with wt or mutant viruses; serum was collected at specific time points (early/acute infection; mid; late/endpoint/chronic infection); and mice were sacrificed at the late/endpoint for full analysis. There were no differences in virus production and egress, matching cell culture infection data. Differential effects on serum and splenic CD4 T-cell depletion showed that the SMR mutation eliminated CD4 depletion during infection. Pro-inflammatory cytokine/chemokine reduction in mutant-versus wt-virus-infected mice highlighted the loss of the “cytokine storm” linked to chronic immune activation (CIA). This agrees with observations in human infections (Konadu et al. 2015b) and links the SMR mutation with CIA. These data suggest that Nef SMR, critical for exNef secretion, modulates HIV pathogenesis and is a likely target for antiviral therapy.

Dr. Kenneth Witwer's group investigated the role of EVs in modulating virus production (Liao et al. 2017) and more recently in mediating the deleterious effects of cigarette smoking. Rates of cigarette abuse are several-fold higher in most HIV-positive populations

compared with uninfected groups. When primary dendritic cells were exposed to cigarette smoke extract (CSE), they produced EVs that in turn provoked inflammatory responses in autologous cells. For example, EVs from CSE-exposed cells pushed Th1/Th17 polarization of CD4+ T cells. These responses were in some cases of a similar magnitude to those induced by direct exposure to CSE. Especially in light of the Haughey group's findings (Dickens et al. 2017) on transfer of EVs between organ systems, these results suggested that EVs contribute importantly to damaging effects of cigarette smoke on end organs in HIV disease.

Conclusions

The panel discussion was moderated by Drs. Yelamanchili and Hu, and during this time, questions from the audience were answered by the panelists. One of the topics discussed was standardized definition, isolation, and characterization of EV subpopulations. EVs have been used as a general term for exosomes, microvesicles, ectosomes, and microparticles based on their biogenesis, physical characteristics (such as size, density, and specific molecules on their surface), or function. It is necessary to clarify the nomenclature in this field to avoid ambiguity. Current efforts aimed at developing and modifying the isolation methods for EV subpopulations and also distinguishing EVs from viral particles were also part of the discussion. Dr. Kashanchi's group developed a method for the isolation of various types of EVs using sucrose density or OptiPrep gradient followed by immunoprecipitation using antibodies to known exosomal markers as reported elegantly in the review by Schwab et al. (Schwab et al. 2015). They were able to distinguish exosomes and virions from HIV-infected cell culture supernatants by a combination of ultracentrifugation and iodixanol (OptiPrep) velocity gradient centrifugation methods (Narayanan et al. 2013). Dr. Bond's group recently described a procedure, also using iodixanol velocity gradients, for the effective isolation and separation of exosomes from HIV-1 virions in human plasma (Konadu et al. 2016). Dr. Pulliam's group successfully isolated neuron-derived exosomes from plasma of HIV-infected subjects using precipitation and immunoabsorption with an antibody to a neuronal specific cell surface marker (Sun et al. 2017). Additionally, Dr. Hill's group reported a novel method for the successful enrichment of exosomes from human brain tissue (Vella et al. 2017). The consensus of the panel was that it is necessary to develop a method for the accurate selection of subpopulations of EVs since the cargo in specific subpopulations of EVs could reflect disease status and could also be used as a diagnostic indicator to determine therapeutic efficacy. Techniques used for the isolation and characterization of EVs have been assessed in many laboratories worldwide and excellently reviewed recently by Dr. Hill's group (Gardiner et al. 2016). Another important issue is to understand how EVs in the circulation system crosstalk with other tissues and organs. Given that both HIV-1 virus and drug abuse have a dramatic impact on immune system including cells in the blood, it was not surprising that plasma EVs contain a variety of biologically active molecules that may enter the CNS and affect brain function and in turn, behavior, as stated by Dr. Buch. An exciting study from Dr. Haughey's group demonstrated that EVs released from the brain travel through the bloodstream to the liver. The EVs then instruct white blood cells to go to the site of injury in the brain (Dickens et al. 2017). The panel agreed that future efforts on "omics" analyses of plasma EVs, especially subpopulations of EVs will be crucial in expanding the

basic understanding of the mechanism(s) regulating communication between the brain and peripheral immune system. While significant progress has been made in this field, efforts are still needed to understand the underlying mechanism(s) by which recipient cells take up EVs. Progress in this area could propel the development of tissue-targeted drug delivery vesicles, techniques for isolation and analyses of single-cell EV protein and RNA, in vitro EV modifications for special applications, and efficient in vivo EV tracking models.

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