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Extracellular vesicles of the blood-brain barrier: Role in the HIV-1 associated amyloid beta pathology

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ABSTRACT

HIV-infected brains are characterized by increased amyloid beta (Aβ) deposition. It is believed that the blood-brain barrier (BBB) is critical for Aβ homeostasis and contributes to Aβ accumulation in the brain. Extracellular vesicles (ECV), like exosomes, recently gained a lot of attention as potentially playing a significant role in Aβ pathology. In addition, HIV-1 hijacks the exosomal pathway for budding and release. Therefore, we investigated the involvement of BBB-derived ECV in the HIV-1-induced Aβ pathology in the brain. Our results indicate that HIV-1 increases ECV release from brain endothelial cells as well as elevates their Aβ cargo when compared to controls. Interestingly, brain endothelial cell-derived ECV transferred Aβ to astrocytes and pericytes. Infusion of brain endothelial ECV carrying fluorescent Aβ into the internal carotid artery of mice resulted in Aβ fluorescence associated with brain microvessels and in the brain parenchyma. These results suggest that ECV carrying Aβ can be successfully transferred across the BBB into the brain. Based on these observations, we conclude that HIV-1 facilitates the shedding of brain endothelial ECV carrying Aβ; a process that may increase Aβ exposure of cells of neurovascular unit, and contribute to amyloid deposition in HIV-infected brain.

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1. Introduction

Increased amyloid beta (Aβ) deposition is common in the brains of HIV-1 infected individuals (Achim et al., 2009; Brew et al., 2009). It is believed that this process contributes to the development of HIV-associated neurocognitive disorders (HAND), as HAND prevalence in older HIV-1-infected patients is associated with early beta-amyloidosis (Xu and Ikezu, 2009; Soontornniyomkij et al., 2012). The most abundant Aβ deposition occurs in the perivascular space, (Green et al., 2005; Xu and Ikezu, 2009; Soontornniyomkij et al., 2012; Steinbrink et al., 2013), suggesting that the brain microvessels may be involved in amyloid pathology. In fact, the blood-brain barrier (BBB) is crucial for Aβ homeostasis, and plays a role in Aβ accumulation in the brain (Deane and Zlokovic, 2007). To support this notion, we demonstrated that HIV-1 could elevate Aβ levels in human brain endothelial cells (HBMEC), and enhance its transendothelial transfer (Andras et al., 2010, 2012).

Extracellular vesicles (ECV) are heterogeneous in their origin, size, and content. ECV include exosomes with a size of approximately 30–100 nm (Mathivanan et al., 2010), and other larger vesicles (Thery et al., 2009; Meckes and Raab-Traub, 2011). Exosomes, are formed in a two-step process involving vesicle budding of the endosomal membranes generating intraendosomal vesicles, followed by the endosome membrane fusion with the plasma membrane which releases these vesicles as exosomes. Their content is diverse and includes mRNAs, miRNAs, lipids and proteins. Exosome cargo can be released into the immediate microenvironment, or at the periphery when exosomes travel via bloodstream. Exosomes being of endosomal origin, contain membrane transport and fusion proteins (e.g., GTPases, annexins, flotillin), tetraspanins (e.g., CD9, CD63, CD81), which are commonly utilized as protein markers for ECV (Vlassov et al., 2012). Exosomes can be taken up into the target cells by endocytosis (Tian et al., 2010).

ECV were recently postulated as having a significant involvement in Aβ pathology, and in various neurodegenerative diseases (Vella et al., 2008; Kalani et al., 2014; Gupta and Pulliam, 2014). In addition, HIV-1 is known to use the exosomal pathway to its advantage to generate infectious particles, and to increase viral spread (Sampey et al., 2014). Although ECV released by human brain microvascular cells (HBMEC) were isolated, characterized (Haqqani et al., 2013), and reviewed before (Andras and Toborek, 2016), to the best of our knowledge, there are no reports on BBB-derived ECV/exosome involvement in Aβ pathology, especially in the context of HIV-1 infection.

In the present study, we hypothesize that BBB-derived ECV are involved in Aβ transfer into the brain, increasing exposure to Aβ of the

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BBB-associated cells which form the neurovascular unit. The results indicate that HIV-1 exposure increases the release of ECV from HBMEC, and enhances their loading with Aβ. Interestingly, HBMEC-derived ECV-Aβ cargo can be successfully transferred to astrocytes and pericytes. In addition, infusion of HBMEC-derived ECV with Aβ into the internal carotid artery of mice resulted in association of Aβ with brain microvessels and its delivery into the brain parenchyma.

2. Materials and methods

2.1. Cell cultures

Human brain microvascular endothelial cells (HBMEC) used in the present study represent a stable, well characterized, and differentiated human brain endothelial cell line (Weksler et al., 2005). Briefly, normal human brain endothelial cells were transduced by lentiviral vectors incorporating human telomerase or SV40T antigen. Among several stable immortalized clones obtained by sequential limiting dilution cloning of the transduced cells, the hCMEC/D3 cell line (referred here as HBMEC) was selected as expressing normal endothelial markers and demonstrating blood-brain barrier characteristics. HBMEC for the present study were supplied by Dr. Couraud (Institut Cochin, Paris, France). Cells were cultured on collagen type I (BD Biosciences Pharmingen, San Jose, CA) coated dishes in EBM-2 medium (Clonetics, East Rutherford, NJ) supplemented with VEGF, IGF-1, EGF, basic FGF, hydrocortisone, ascorbate, gentamycin, and 0.5% exosome depleted fetal bovine serum (Exo-FBS; Systems Bioscience, Mountain View, CA).

Human brain astrocytes (SVG astroglia) were purchased from American Type Culture Collection (ATCC, Manassas, VA). They were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% Exo-FBS (Systems Biosciences), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C and 5% CO2.

Human brain pericytes were purchased from ScienCell (Carlsbad, CA) and maintained in Pericyte Medium (ScienCell) supplemented with Pericyte Growth Supplement (PGS, ScienCell), penicillin/streptomycin solution (P/S, ScienCell), and 20% exosome depleted fetal bovine serum (Exo-FBS; Systems Bioscience) at 37 °C and 5% CO2.

2.2. HIV infection and Aβ treatment

HIV stock was generated using human embryonic kidney (HEK) 293T cells (ATCC) transfected with pYK-JRCSF plasmid containing full-length proviral DNA. Throughout the study, HBMEC were exposed to HIV particles at the p24 level of 30 ng/ml as previously reported (Andras and Toborek, 2014). Treatment was terminated by the removal of cell culture media for ECV isolation.

Aβ (1–40) HiLyte was purchased from Anaspec (San Jose, CA). Aβ (1–40) was dissolved in sterile ultra-pure water (ELGA Purelab Classic, Lowell, MA). Freshly solubilized Aβ solutions without pre-aggregation were used for experiments because this form of Aβ was demonstrated to induce proinflammatory reactions in isolated rat brain microvessels (Paris et al., 2002). Aβ (1–40) HiLyte was dissolved first in a basic buffer (0.1 M NH_4OH), and then further diluted in PBS as suggested by the manufacturer. Cells were treated with Aβ (1–40) or Aβ (1–40) HiLyte at the concentration of 100 nM for 48 h in complete medium. Although uptake of Aβ by the BBB occurs rapidly (Yamada et al., 2008), we terminated the treatment at 48 h to allow more ECV to be secreted into the culture medium.

2.3. Transfection of brain endothelial cells and ECV isolation

HBMEC were transfected with the CD63 and CD9 Cyto-Tracer constructs (pT CD63-GFP, pT CD9-RFP, respectively) and the non-targeting construct, pT-CYTO RFP (all from Systems Bioscience) using Purefection Transfection Reagent following the manufacturer's protocol. Twenty four hours post transfection, cells were exposed to HIV-1 or/and Aβ (1–40) HiLyte for 48 h.

ECV isolation was performed using ExoQuick-TC exosome precipitation solution (System Bioscience) according to the manufacturer's specifications. Briefly, 10 ml culture medium was mixed thoroughly with 2 ml of Exo-Quick exosome precipitation solution and incubated overnight at 4 °C. The next day, samples were centrifuged at 1500*g* for 30 min, the supernatant was removed, and centrifuged again at 1500*g* for 5 min. The ECV pellet was resuspended in PBS and used for further studies.

2.4. ECV infusion surgery

All in vivo experiments were performed according to the guidelines of the American Association for Accreditation of Animal Care (AAALAC), and were approved by the University of Miami Institutional Animal Care and Use Committee. Male C57BL/6 mice of 10 to 12 weeks of age (Jackson Laboratory, Bar Harbor, ME) were weight-matched, and randomly assigned to different treatment groups. Mice were anesthetized with isoflurane, and infused with ECV isolated either from Aβ-exposed HBMEC or saline-treated HBMEC into the brain circulation via the internal carotid artery (ICA), as previously described (Chen et al., 2009). Briefly, the common carotid artery (CCA) was exposed until its bifurcation, where the external carotid artery (ECA) and the ICA begin. Using nylon sutures, a permanent knot was placed at the highest point possible of the ECA, and a removable knot was placed at the lowest point possible of the ECA, immediately above the CCA bifurcation. Next, the CCA and the ICA were temporarily closed using vessel clips. A small incision in the ECA, between the two knots, was performed, where a capillary tubing attached to a syringe containing the ECV solution was inserted. After removing the vessel clip from the ICA, the solution was slowly infused. Mice were euthanized 1 h post infusion, perfused with saline, followed by decapitation, and brain tissue harvesting for microvessel isolation and immunofluorescence, as described below.

2.5. Brain microvessel isolation

Brain microvessels were isolated from the ipsilateral hemisphere as previously described, with few modifications (Park et al., 2013). Briefly, dissected brains were homogenized in 4 ml of ice-cold isolation buffer (102.0 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 15 mM HEPES, 25 mM $NaHCO_3$, 10 mM glucose, 1 mM Na pyruvate; pH 7.4 with Halt™ protease inhibitor cocktail [Thermo Fisher Scientific, Waltham, MA]). Samples were then filtered through a 300 μm mesh filter (Spectrum Laboratories, Rancho Dominguez, CA), and transferred to a centrifuge tube containing 8 ml of 26% dextran (M.W. 75,000) in isolation buffer solution; followed by centrifugation at 5800g, 20 min, at 4 °C. After discarding the supernatant, the pellet was resuspended in 2 ml of isolation buffer, filtered through a 120 μm mesh filter (Millipore Sigma, Billerica, MA), and submitted for another round of centrifugation at 1500*g*, for 10 min, at 4 °C. The supernatant was removed, and the pellet was resuspended in 200 μl of isolation buffer. The microvessel-enriched fraction was smeared onto a glass slide (approximately 7 μl per slide), and heat-fixed at 98 °C for 10 min.

Slides containing the isolated brain microvessels were washed twice with PBS, incubated for 5 min at room temperature, and stained with DAPI (4′,6-Diamidino-2-Phenylindole, Dihydrochloride, Thermo Fisher Scientific) at 1 μg/ml concentration in PBS. Slides were mounted with ProLong Diamond Antifade Mountant (Thermo Fisher Scientific) and imaged on Olympus Fluoview 1200 confocal microscope using a 60× oil immersion lens.

2.6. Nanoparticle tracking analysis (NTA)

ECV were analyzed by NanoSight model NS300 (Malvern Instruments Company, Nanosight, Malvern, United Kingdom). Briefly, ECV

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