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The HBx gene of hepatitis B virus can influence hepatic microenvironment via exosomes by transferring its mRNA and protein

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ABSTRACT

The cellular secretory vesicles known as 'exosomes' have emerged as key player in intercellular transport and communication between different eukaryotic in order to maintain body homeostasis. Many pathogenic viruses utilize exosome pathway to efficiently transfer bioactive components from infected cells to naïve cells. Here, we show that HBx can tweak the exosome biogenesis machinery both by enhancing neutral sphingomyelinase2 activity as well as by interacting with exosomal biomarkers such as neutral sphingomyelinase2, CD9 and CD81. The nano particle tracking analysis revealed enhanced secretion of exosomes by the HBx-expressing cells while confocal studies confirmed the co-localization of HBx with CD9 and CD63. Importantly, we observed the encapsulation of HBx mRNA and protein in these exosomes besides some other qualitative changes. The exosomal cargo secreted by HBx-expressing cells had a profound effect on the recipient hepatic cells including creation of a milieu conducive for cellular-transformation. Thus, the present study unfolds a novel role of HBx in intercellular communication by facilitating horizontal transfer of viral gene products and other host factors via exosomes in order to support viral spread and pathogenesis.

1. Introduction

Exosomes are nano-sized particles shed in the extracellular milieu by most cells. Exosomes are present in nearly all body fluids and are known to carry various bioactive molecules including proteins, DNA, mRNAs, and non-coding RNAs (reviewed in Braicu et al., 2015; Colombo et al., 2014). These molecules are specifically sorted and loaded in exosomes during its biogenesis (Dreyer et al., 2016; Trajkovic et al., 2008). Exosomes are now recognized as a new class of intercellular communicators that allow horizontal transfer of information to recipient cells by virtue of their cargo both in health and disease (reviewed in Ratajczak and Ratajczak, 2016; Regev-Rudzki et al., 2013; Schorey et al., 2015; Tkach and Théry, 2016). For example, exosomes derived from hepatocytes are able to drive the cellular machinery to produce sphingosine-1-phosphate in target hepatocytes resulting in cell proliferation and liver regeneration (Nojima et al., 2016). Besides, an active communication between stromal and parenchymal cells holds the key for maintenance of tissue homeostasis which when perturbed can create a microenvironment conducive for diseased conditions (Syn et al., 2016). Likewise, the hepatic stellate cells (HSCs) that are known to contribute towards hepatic fibrosis, are activated by neighbouring parenchymal, non-parenchymal or hepatic immune cells via a paracrine mechanism (Greuter and Shah, 2016). The hepatic fibrosis subsequently progresses to irreversible liver cirrhosis and/or hepatocellular carcinoma. Increasing evidence suggests that exosomes also play a critical role in cancer development and metastasis by modulating the genetic and epigenetic events related to cancer (Melo et al., 2014; Soung et al., 2016). The altered production of exosomes and aberrant exosomal contents could reflect the pathological state of the body. Therefore, the biologically active cargo of exosomes could be a potential source of circulating biomarkers and therapeutics (Ko et al., 2016; Schey et al., 2015; Sheridan, 2016).

Exosomes and cell-to cell communication are also reported to be play an important role in immune regulation and antiviral response during viral infection (Alenquer and Amorim, 2015). Human pathogenic viruses such as human immunodeficiency virus-1 (Madison and Okeoma, 2015; Wiley, 2006), human papillomavirus (Honegger et al., 2013) and members of herpes virus family such as human herpes virus

Abbreviations: PBS, phosphate buffer saline; EGFR, epidermal growth factor; αSMA, α smooth muscle actin; TGFβ, transforming growth factor β; col1a, collagen 1A; col3A, collagen 3A; MBP, maltose binding protein; BSA, bovine serum albumin; D2O, deuterium oxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide; ng, nanogram; EDTA, ethylenediaminetetraacetic acid

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(Mori et al., 2008), herpes simplex virus (Kalamvoki and Deschamps, 2016) and Epstein-Barr virus (Canitano et al., 2013) have been shown to use exosomal secretory pathways in disease pathogenesis and viral spread. Exosomes derived from the blood of hepatitis C virus (HCV)-infected patients have been shown to carry viral RNA and host proteins Ago2 and HSP90 which facilitate viral transmission to naïve cells and remains unaffected in the presence of neutralising antibodies (Bukong et al., 2014; Ramakrishnaiah et al., 2013). However, HCV replication can be inhibited by using exosomes isolated from interferon-stimulated liver endothelial cells suggesting transfer of some antiviral activity through exosomes (Giugliano et al., 2015). It has been observed that interferon-alpha stimulated liver cells secrete exosomes that can attenuate hepatitis B virus (HBV) replication (Li et al., 2013). Further, the core protein of HCV can enhance the crosstalk between hepatocytes and stromal environment via exosomes by activating TGFβ signalling pathway (Benzoubir et al., 2013) while exosomal microRNAs derived from hepatic tumour cells can regulate healthy liver cells by autocrine and paracrine mechanisms (Kogure et al., 2011). However, the role of exosomes in HBV life cycle and associated liver diseases is poorly understood.

Chronic infection with HBV is a major cause of liver fibrosis, eventually leading to cirrhosis and hepatocellular carcinoma (HCC). The pleiotropic HBx protein of HBV is known to abet oncogenic activities by multiple mechanisms including stimulation of host genes, mitogenic signalling, and interference with cell cycle, proteasomal machinery, ribosome biosynthesis (reviewed in Kumar and Sarkar, 2004; Slagle and Bouchard, 2016). Besides, HBx is reported to regulate the neighbouring hepatic cells by paracrine mechanisms (Martín-Vílchez et al., 2008). However, there is no evidence suggesting the involvement of HBx in exosome biogenesis or alteration of its molecular cargo. Now we show that both HBx mRNA and protein get encapsulated in exosomes and shed in the extracellular milieu. The exosomal cargo secreted by the HBx-expressing cells is qualitatively as well as quantitatively different. Further, these exosomes can induce proliferative signalling in HSCs which may contribute towards HBV-associated liver diseases.

2. Materials and methods

2.1. Antibodies and chemicals

Antibodies against CD63 was procured from BD Biosciences, while antibodies against CD81, PCNA, pAkt, Alix, nSMnase2, HBx, and GAPDH were obtained from Santa Cruz Biotechnology (USA). Antibodies against CD9, c-Met and αSMA were from EMD Millipore (USA) whereas 10 nm colloidal gold-conjugated Protein A was purchased from Genetix Biotech, India. RNase A and yeast tRNA (both 10 mg/ml) and DNAse I (1 unit/μl) were procured from Thermo Fisher Scientific, USA.

2.2. Plasmids, cell culture and transfection

Huh7 cells were kindly provided by Dr Aleem Siddiqui (University of Colorado, Denver). The cultures were maintained and transfected as described earlier (Kapoor et al., 2013). Cells were put in exosome isolation media 24 h post transfection. LX-2 cells were a kind gift from Dr. Scott Friedman (Mount Sinai School of Medicine, New York, NY) and were maintained in DMEM supplemented with 2% FBS. The HBx expression plasmid, X0 and the vector control (pSG5) have been described earlier (Kumar et al., 1996). The expression and purification of Maltose-binding protein (MBP) and recombinant HBx protein (X0-MBP) are described elsewhere (Sidhu et al., 2014). pGFP-HBx was procured from Addgene (Addgene plasmid #24931). The nSMnase2 expression plasmid was a kind gift from Dr. Takahiro Ochiya (Kosaka et al., 2010).

2.3. Confocal microscopy

Huh7 cells were seeded and transfected with pGFP-HBx on 8 well chambered slides (SPL Life Sciences, Korea). Media was aspirated after 48 h and cells were washed once with 1 x PBS and fixed with 4% paraformaldehyde. Permeabilization was done with 0.1% triton X-100 in 1 x PBS at RT followed by blocking with 10% serum in PBS. Cells were incubated with primary antibody against CD81 or CD63 at a dilution of 1:250 in blocking solution followed by secondary Alexa fluor 594 goat anti mouse IgG (H + L) (Molecular probes, Life Technologies, USA). The slides were mounted in ProLong Gold anti-fade reagent with DAPI (Molecular Probes, Life Technologies, USA) and images were captured at 60 x magnification in Nikon A1R confocal microscope and images were processed and co-localisation coefficient was derived using NIS elements AR version 3.0 (Nikon, Japan).

2.4. Exosome isolation and purification

Exosomes were isolated from conditioned media as described by Kosaka et al. (2010). Briefly, cells were cultured in 1 x DMEM (Gibco BRL) with 10% FBS for 24 h post transfection. Cells were washed twice with large volumes of 1 x PBS [137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO4 and 1.47 mM KH_2PO4) in H_2O, pH adjusted to 7.4] and then were maintained in 1 x Advanced medium (Gibco BRL) for 48–72 h. The conditioned media from the cell lines was collected and spun at 2000 × *g* for 15 min at room temperature (RT) and then at 12,000 × *g* for 35 min at RT. The medium was filtered through a 0.22 μ filter and then ultra-centrifuged at 110,000*g* for 70 min at 4 °C. The pellets was washed with 1 x PBS followed by ultracentrifugation as above. The final exosome pellet was re-suspended in either 1 x PBS or Assay buffers and its protein content was measured by Bradford assay.

The exosomes were also isolated using Total Exosome Isolation Solution (TEIS) (Invitrogen, Carlsbad, California) as per manufacturer's instructions. Briefly, after the filtration, the conditioned medium was mixed with TEIS in 2:1 ratio and left overnight at 4 °C with gentle agitation. The sample was then spun at 10000 x g for 60 min at 4 °C and the supernatant was discarded. The pellets was re-suspended in 1xPBS followed by spin once again as above. The final pellet was re-suspended either in 1xPBS or in 500 μl of Trizol reagent (Ambion, Life Technologies, USA) as per the experimental requirements.

2.5. Sucrose cushion purification

Floatation of exosomes on sucrose cushion was essentially done as described by Théry et al. (2006). Briefly, the exosome pellet collected after ultracentrifugation step as described above, was suspended in ~28 ml of 1xPBS and was gently overlaid on a 4 ml cushion of Tris/Sucrose/D_2O in a pollyallomer tube and spun in SW 32 rotor (Beckman Coulter, USA) at 1,10,000 x g for 70 min at 4°C. The tube was punctured from the side with a syringe fitted with 18 gauge needle and ~3.5 ml of the Tris/sucrose/D_2O cushion was collected. The sample volume was made up to 11 ml and spun again under same conditions in a SW41 rotor. The pellet obtained after this spin was collected and re-suspended in appropriate assay buffers.

2.6. Incubation of hepatic stellate cells with exosomes

LX-2 cells were seeded in 12 well plates. After 24 h, cells were thoroughly washed with 1 x PBS to remove any serum-associated exosomes and incubated in Advanced media for next 24 h. Cells were then incubated with purified exosomes (~1 μg protein) for 24 h and the harvested for western blot or qRT-PCR analysis.

2.7. Western blotting and immuno-precipitation

Exosome samples or cells lysates were denatured by boiling in 4 x