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Research Paper

HIV Activates the Tyrosine Kinase Hck to Secrete ADAM Protease-Containing Extracellular Vesicles

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ABSTRACT

HIV-Nef activates the myeloid cell-typical tyrosine kinase Hck, but its molecular role in the viral life cycle is not entirely understood. We found that HIV plasma extracellular vesicles (HIV pEV) containing/10 proteases and Nef also harbor Hck, and analyzed its role in the context of HIV pEV secretion. Myeloid cells required Hck for the vesicle-associated release of ADAM17. This could be induced by the introduction of Nef and implied that HIV targeted Hck for vesicle-associated ADAM17 secretion from a myeloid compartment. The other contents of HIV-pEV, however, including miRNA and effector protein profiles, as well as the presence of haptoglobin suggested hepatocytes as a possible cellular source. HIV liver tissue analysis supported this assumption, revealing induction of Hck translation, evidence for ADAM protease activation and HIV infection. Our findings suggest that HIV targets Hck to induce pro-inflammatory vesicles release and identifies hepatocytes as a possible host cell compartment.

1. Introduction

One of the well documented effects of HIV Nef is the activation of the tyrosine kinase Hck by interaction of its PxxP motif with the Hck SH3 domain (Saksela et al., 1995). This opens an intramolecular lock and constitutes the first step in the activation of the kinase (Lee et al., 1995, 1996; Moarefi et al., 1997). While the PxxP motif seemed required for disease progression in animal models (Khan et al., 1998) and humans (Trible et al., 2007), the molecular role of Hck in the viral life cycle remained unclear (Saksela, 2011). Hck is expressed predominantly in myeloid cells but not T cells, the main host cell of HIV. It was therefore speculated that in T cells Nef may recruit another SH3 domain-containing protein; however, the identity of this protein is still a matter of debate (Saksela, 2011).

In transient transfection systems Nef-induced Hck activation dysregulated signaling at the Golgi apparatus (Hassan et al., 2009; Hiyoshi et al., 2012), consistent with reports suggesting that secretory membrane trafficking from the Golgi is regulated by Src kinases (Sallese et al., 2009). In line with such a function, Hck augmented production and release of pro-inflammatory cytokines like TNF (English et al., 1993; Ernst et al., 2002). ProTNF is processed also in Golgi-derived compartments after PMA stimulation or Nef-induced ADAM17 activation, and is secreted in vesicles via membrane protrusions (Ostalecki et al., 2016). Such protrusions were also observed following Hck activation (Carreno et al., 2002).

A number of reports demonstrated HIV replication in liver cells in vitro and in vivo (Blackard and Sherman, 2008; Cao et al., 1992, 1990; Housset et al., 1993; Tuyama et al., 2010) and HIV infection is frequently associated with liver disorders (Mendeni et al., 2011; Price et al., 2012). HIV-induced immunosuppression and the risk of liver-related death correlate strongly, and liver-related morbidity is the most frequent cause of death in chronic infection (Towner et al., 2012; Weber et al., 2006). At present, however, HIV liver infection is not considered to be relevant for disease pathogenesis.

We recently reported that the number of ADAM17/Nef-containing plasma extracellular vesicles (pEV or HIV pEV) is strongly upregulated in HIV infection. Their number did not decline during therapy and their ADAM17/Nef content correlated inversely with CD4 T cell counts. Hence identifying the compartment shedding HIV pEV was of considerable importance. Preliminary findings had suggested that they did not derive from T cells (Lee et al., 2016). In this report we aimed at identifying their cellular origin, analyzing the factor content in HIV pEV. The

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detection of Hck not only explained a possible role of the tyrosine kinase in HIV biology, but also pointed at myeloid cells and hepatocytes as the likely cellular origin of HIV pEV.

2. Materials and Methods

2.1. Cell Lines

Liver cell lines Huh7 and Sk-Hep-1 (kindly provided by P. Knolle, Technische Universität München) were grown in DMEM (Sigma-Aldrich) supplemented with 10% Fetal calf serum (FCS, Sigma-Aldrich) and 1% penicillin-streptomycin (Lonza). Sk-Hep1 cells were additionally maintained in 40 μM β-mercaptoethanol (Carl Roth). LX-2 cells were provided by SL. Friedman (Icahn School of Medicine) and cultured in DMEM high glucose (Life Technologies) supplemented with 2% FCS, 1% penicillin-streptomycin. All cells were grown at 37 °C under 5% CO_2.

2.2. EV Depletion of FCS and Human Serum for Cell Culture

To assure that EV generated from cell culture were not contaminated by outside sources, heat inactivated FCS and human serum for medium supplementation were depleted of bovine EV by ultracentrifugation for 18 h at 110,000*g*, 4 °C before use.

2.3. Isolation and Purification of EV

EV purification was performed essentially as described previously (Lee et al., 2016). Briefly, supernatants were collected after 48 h and centrifuged for 20 min at 2000*g*, 30 min at 10,000*g* and ultra-centrifuged for 1 h at 100,000*g*. Pellets were resuspended in 35 ml PBS and centrifuged at 100,000*g* for 1 h. Pellets were resuspended in 100 μl PBS and considered as EV preparations.

For EV purification from patient samples, 30 ml blood plasma was diluted with 30 ml PBS and centrifuged for 30 min at 2000*g*, 45 min at 12000*g* and ultra-centrifuged for 2 h at 110,000*g*. Pellets were resuspended in 30 ml PBS and centrifuged at 110,000*g* for 1 h. Pellets were again resuspended in 100 μl PBS and considered as EV preparations. For further purification, EV were diluted in 2 ml of 2.5 M sucrose, 20 mM Hepes/NaOH, pH 7.4 and a linear sucrose gradient (2–0,25 M sucrose, 20 mM Hepes/NaOH pH 7.4) was layered on top of the EV suspension. The samples were then centrifuged at 210,000*g* for 15 h. Gradient fractions were collected and the refractive index was determined. Each fraction was diluted in 10 ml PBS and ultra-centrifuged for 1 h at 110,000*g*. Pellets were solubilized in SDS sample buffer or resuspended in 100 μl PBS and analyzed by immunoblotting or Cytokine/Chemokine/soluble Factor (CCF) protein array (see Supplementary information).

To validate our centrifugation-based pEV isolation protocol, we generated an EV spike-in control (from a stable cell line producing EV), containing an EBV-derived miRNA (BHRF1-2*) that was not found in human pEV-miRNAs, but was detectable by the miRNA microarray (Agilent). After spike-in, BHRF1-2* miRNA was readily detected with comparable efficiency in 4 different plasma samples (data not shown).

2.4. Peripheral Blood Mononuclear Cell (PBMC) Preparation

Leukoreduction system chambers (LRSCs) (Pfeiffer et al., 2013) from healthy donors were acquired after plateletpheresis. The resulting platelet free cell sample was diluted 1:2 in PBS and the PBMC containing buffy coat was isolated after density gradient centrifugation on Lymphoprep (Axix Shield 1114544) at 500*g* for 30 min at room temperature. PBMCs were then washed 3 times in PBS/1 mM EDTA; 1. wash: 282 g, 15 min, 4 °C; 2. wash: 190 g, 10 min, 4 °C; 3. wash: 115 g, 12 min, 4 °C.

2.5. Generation of Immature/Mature Dendritic Cells (DC)

PBMCs were isolated from LRSCs as described above, resuspended in 1× BD IMag Buffer (BD Biosciences 552362) and counted. Monocytes were then isolated from 1.5×10^7 PBMCs using BD IMag Anti-Human CD14 Magnetic Particles (BD Biosciences 557769) according to the manufacturer's instructions. 6.0×10^6 monocytes per well were then seeded in a 6 well plate in RPMI supplemented with 1% heat inactivated human serum from human male AB plasma (Sigma-Aldrich). Monocyte-derived DC were generated supplementing the medium with 800 IU/ml of recombinant GM-CSF and 250 IU/ml of recombinant IL-4 (both from CellGenix) on day 1 after isolation and 400 IU/ml of recombinant GM-CSF and 250 IU/ml of recombinant IL-4 on days 3, 5 and 6. For EV isolation from immature DC, cells were washed with PBS on day 7 and 10 ml RPMI containing 1% of EV-depleted, heat-inactivated human serum and 1% of penicillin/streptomycin was added. After 24 h the supernatant was harvested. For EV isolation from mature DC, immature DC cultures were supplemented for 24 h with a maturation cocktail 200 IU/ml IL-1ß, 1000 IU/ml IL-6 (both from CellGenix), 10 ng/ml TNF (beromun; Boehringer Ingelheim) and 1 μg ml^{-1} Prostin E2 (PGE2, Pfizer). Subsequently cells were washed 1 time with PBS and seeded in 10 ml of RPMI supplemented with 1% of heat-inactivated and EV-depleted serum and 1% of penicillin/streptomycin. After additional 24 h the supernatant was harvested. EV from immature and mature DC were purified as described above.

2.6. Generation of Macrophages

PBMCs were isolated from LRSCs as described above. Monocytes were separated from the non-adherent fraction (NAF) by plastic adherence on cell culture flasks and cultured in RPMI supplemented with 1% human serum and 1% of penicillin/streptomycin. On days 1, 3, 5, 7 and 9 after seeding, medium was supplemented with 800 IU/ml of GM-CSF. On day 11, medium was removed, cells were washed with PBS and 20 ml of RPMI supplemented with 1% of EV depleted human serum and 1% of penicillin/streptomycin was added. After 24 h supernatant was harvested and EV were isolated as described above.

2.7. Generation of Primary Myeloid Cells (Adherent PBMC)

PBMCs were isolated from LRSCs as described above. Monocytes were separated from the non-adherent fraction (NAF) by plastic adherence on cell culture flasks and cultured in RPMI supplemented with 1% human serum and 1% of penicillin/streptomycin. On day 1 after seeding, medium was supplemented with 800 IU/ml of recombinant GM-CSF and 250 IU/ml of recombinant IL-4 (both from CellGenix). After 24 h supernatant was harvested and EV were isolated as described above.

2.8. Nef Antibodies and Detection Reagents

Different anti-Nef antibodies and reagents were used: (1) anti-Nef JR6, a mouse monoclonal antibody (Abcam ab42358); (2) anti-Nef 2A3, a mouse monoclonal antibody (Abcam ab77172); (3 and 4) anti-Nef sheep serum, either as a purified biotinylated polyclonal antibody or non-labeled (both from Targeted Affinity Oy, Helsinki); (5) anti-Nef polyclonal serum (provided by Mark Harris, Leed University). All Nef-antibodies were used to demonstrate the presence of Nef in pEV. For immunoblotting JR6 turned out to have the highest sensitivity and specificity as judged by the ratio of Nef vs. background staining. For detection in tissue we used the biotinylated anti-Nef sheep serum and the JR6 antibody.

2.9. Antibodies

The following antibodies were used for immunostaining or immunoblotting: anti-ADAM10 (mouse monoclonal, Abcam ab73402), anti-

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