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Original Articles

Induction of hyaluronan production by oncogenic KSHV and the contribution to viral pathogenesis in AIDS patients

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ABSTRACT

Kaposi sarcoma-associated herpesvirus (KSHV) is the etiologic agent for Kaposi's sarcoma (KS) and primary effusion lymphoma (PEL), malignancies arising primarily in immunocompromised patients particularly AIDS-patients, which still lack effective therapy. Hyaluronan (HA) is a large glucuronic acid and has been found closely related to multiple functions in cancer cells, although its role in viral oncogenesis remains largely unknown. Here we provide first evidence that KSHV *de novo* infection induces HA production from primary endothelial cells through upregulation of HA synthase gene 1 (*Has1*) and a multifunctional glycoprotein, CD147. Further data demonstrate that KSHV-induced HA production requires viral latent protein, LANA (in particular functional domain A) and MAPK/ERK signaling activities. In functions, HA production is necessary for KSHV/LANA-induced primary endothelial cell invasion, a hallmark feature for KS development. For clinical relevance, our data indicate that the KSHV+ group has higher levels of HA and Has1 activities in its plasma than the KSHV– group of cohort HIV-infected patients. Together, our findings provide innovative insights into the mechanisms of oncogenic virus activation of HA production and its role in virus-associated malignancy pathogenesis, which may help to develop novel therapeutic strategies by targeting HA and related signaling.

Introduction

Hyaluronan (HA) is a very large, linear glycosaminoglycan composed of repeating disaccharides of glucuronic acid and *N*-acetylglucosamine [1]. In addition to its structural role through interaction with other extracellular matrix (ECM) components, HA binds to several cell surface receptors such as CD44, LYVE-1 and RHAMM that induce the transduction of a range of intracellular signals and contribute to multiple cellular functions such as embryonic development, healing processes and inflammation [2]. HA is overproduced by many types of tumors, and in some cases, HA levels are prognostic for malignant progression [3]. Moreover, HA and related signaling transductions have been involved in many malignant behaviors of cancer cells, including migration/invasion, angiogenesis, epithelial–mesenchymal transition (EMT), multidrug resistance, and metastasis [4–6]. Currently, ~20% of human cancers have been attributed to virus infection [7]; however, there are limited data describing the role of HA production in viral oncogenesis or how oncogenic viral proteins regulate HA level and related signaling transductions.

Kaposi sarcoma-associated herpesvirus (KSHV) is the etiologic agent for Kaposi's sarcoma (KS) and primary effusion lymphoma (PEL), malignancies arising primarily in patients infected with the human immunodeficiency virus (HIV) or in those receiving organ transplants [8,9]. Furthermore, despite the reduced incidence of KS in the era of highly active antiretroviral therapy (HAART) for HIV infection, KS still remains the most common Acquired immunodeficiency syndrome (AIDS)-associated tumor and a leading cause of morbidity and mortality in this setting [10]. Another KSHV-caused malignancy, PEL, comprises transformed B cells harboring KSHV and arises preferentially within the pleural or peritoneal cavities of immune-suppressed patients [9]. PEL is a rapidly progressing malignancy with a median survival time of approximately 6 months, even under the combinational chemotherapy [11]. Our recent study demonstrates that the glycoprotein, CD147, interacts with the lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) and the drug transporter, breast cancer resistance protein (BCRP)/ABCG2, to promote multidrug chemoresistance in KSHV+ PEL cells [12]. Moreover, we found higher

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levels of HA and HA synthase gene (*Has1–3*) transcripts in chemoresistant PEL cell-lines than in chemosensitive ones. In addition, small HA oligosaccharides (oHA) that interact monovalently with HA receptors, and competitively blocking polyvalent interactions between receptors and endogenous HA, sensitize drug resistant PEL cells to chemotherapeutic agents [12]. In the current study, we investigate the role of HA production in KSHV-infected primary endothelial cells, which represent the major cellular component of KS tumors, and identify the underlying mechanisms whereby oncogenic viral proteins regulate HA production.

Materials and methods

Cell culture, reagents and infection protocol

KSHV-infected PEL cells (BCBL-1) were kindly provided by Dr. Dean Kedes (University of Virginia) and maintained in RPMI 1640 medium (Gibco) with supplements as described previously [12]. Human umbilical vein endothelial cells (HUVEC) were grown in DMEM/F-12 50/50 medium (Cellgro) supplemented with 5% FBS. Selective inhibitors targeting the mitogen-activated protein kinase (MEK; U0126) and NF-κB (Bay11-7082) were purchased from Sigma. Hyaluronan oligosaccharides (oHA) were prepared as described previously [13]. To obtain KSHV for infection experiments, BCBL-1 cells were incubated with 0.6 mM valproic acid for 6 days, purified virus concentrated from culture supernatants and infectious titers were determined as described previously [14].

Cell transfection

HUVEC were transfected with control vector pcDNA3.1, pcDNA3.1-LANA (pcLANA) or LANA deletion fragments (pcLANA-A, pcLANA-AB, pcLANA-AC, pcLANA-BC and pcLANA-C) or pcDNA3.1-ERK (pcERK) in 12-well plates for 48 h using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Transfection efficiency was determined through co-transfection of a lacZ reporter construct and quantified as described previously [14]. For RNA interference assays, *Has1* or *CD147* ON-TARGET plus SMART pool siRNA (Dharmacon), or negative control siRNA, was delivered using the DharmaFECT transfection reagent according to the manufacturer's instruction.

Immunofluorescence assays

Cells were incubated in 1:1 methanol–acetone at 20 °C for fixation and permeabilization, followed by a blocking reagent (10% normal goat serum, 3% bovine serum albumin, and 1% glycine) for an additional 30 min. Cells were then incubated for 1 h at 25 °C with 1:1000 dilution of a rat anti-LANA monoclonal antibody (ABI, for LANA wt) or a mouse anti-V5-Tag monoclonal antibody (Cell Signaling, for LANA deletion fragments) followed by 1:100 dilution of a goat anti-rat or goat anti-mouse secondary antibody conjugated to Texas Red (Invitrogen). For intracellular HA detection, cells were permeabilized for 20 min at room temperature with 0.1% Triton-X-100 in 1% BSA, and incubated overnight at 4 °C with bHABC (biotinylated hyaluronan binding complex, Sigma) (1.25 μg/mL) in 1% BSA. To remove the pericellular HA, fixed cells were treated with Streptomyces hyaluronidase (1 turbidity reducing unit/mL, Seikagaku Kogyo) before permeabilization. After washing, the cells were incubated for 1 h with Alexa 488-labeled streptavidin (1:1000) (Invitrogen) for bHABC staining. Cells were counterstained with 0.5 μg/mL 4′,6-diamidino-2-phenylindole (DAPI, Sigma) in 180 mM Tris–HCl (pH 7.5) for nuclear localization. Slides were washed once in 180 mM Tris–HCl for 10 min and prepared for visualization using a Leica TCPS SP5 AOBS confocal microscope.

Immunoblotting

Total cell lysates (20 μg) were resolved by 10% SDS–PAGE, transferred to nitrocellulose membranes, and immunoblotted with antibodies for CD147 (BD), LANA (ABI), phospho-p44/42 ERK (Thr202/Tyr204), t-p44/42 ERK (Cell Signaling) and β-Actin (Sigma) for loading controls. Immunoreactive bands were identified using an enhanced chemiluminescence reaction (PerkinElmer), and visualized by autoradiography.

Transwell invasion assays

Matrigel Invasion Chambers (BD) were hydrated for 4 h at 37 °C with culture media. Following hydration, media in the bottom of the well was replaced with fresh media, then 2×10^4 HUVEC were plated at the top of the chamber. After 24 h, cells were fixed with 4% formaldehyde for 15 min at room temperature and chambers rinsed in PBS prior to staining with 0.2% crystal violet for 10 min. After washing the chambers, cells at the top of the membrane were removed and cells at the bottom of the membrane counted using a phase contrast microscope. Relative invasion was determined for cells in experimental groups as follows: relative invasion = # invading cells in experimental group/# invading cells in control groups.

ELSA for HA

Concentrations of HA in culture supernatants or plasma from patients were determined using HA ELSA kit (Echelon) according to the manufacturers' instructions.

qRT-PCR

Total RNA was isolated from infected or uninfected cells using the RNeasy Mini kit according to the manufacturer's instructions (QIAGEN). cDNA was synthesized from equal total RNA using SuperScript III First-Strand Synthesis SuperMix Kit (Invitrogen) according to the manufacturer's procedures. The primers designed for target genes are displayed in Supplemental Table 1. Amplification experiments were carried out using an iCycler IQ Real-Time PCR Detection System, and cycle threshold (Ct) values were tabulated in duplicate (cDNA) for each gene of interest for each experiment. "No template" (water) controls were also used to ensure minimal background contamination. Using mean Ct values tabulated for different experiments and using Ct values for β-actin as loading controls, fold changes for experimental groups relative to assigned controls were calculated using automated iQ5 2.0 software (Bio-rad).

Patients and ethics statement

The study was approved by the Institutional Review Board for Human Research (IRB, No. 8079) at Louisiana State University Health Science Center – New Orleans (LSUHSC-NO). All subjects were provided written informed consent. In the current study, a total of 28 HIV+ patients with antiretroviral treatment (ART) in our HIV Outpatient (HOP) Clinic are involved. There are 15 females and 13 males, the average age is 48.6 y (range 21–65 y). The average CD4 T cell count is 539/mL (range 35–1773/mL), and the average HIV viral loads is 5928 copies/mL (range 25–66681 copies/mL).

Plasma and PBMC preparation

Whole blood was collected in heparin-coated tubes, and peripheral blood mononuclear cells (PBMCs) were isolated over a Ficoll-Hypaque cushion. Plasma was isolated by centrifugation. The KSHV infection status was determined by using quantitative ELISAs for identifying circulating IgG antibodies to KSHV proteins (LANA and K8.1) as previously described [15,16].

Statistical analysis

Significance of differences between experimental and control groups was determined using the two-tailed Student's t-test (Excel 8.0). The linear analyses were determined using SPSS Statistics 20.0.

Results

KSHV de novo *infection induces HA production from primary endothelial cells through CD147*

By using an enzyme-linked sorbent assay (ELSA), we found that KSHV *de novo* infection induced a significant increase in extracellular HA produced by human umbilical vein endothelial cells (HUVEC); accumulation of extracellular HA continued for at least 96 h post-infection (Fig. 1A). Furthermore, immunofluorescence assay (IFA) data indicated that KSHV *de novo* infection also induced intracellular HA accumulation within the HUVEC, when compared to uninfected mock cells (Fig. 1B). There are three human HA synthase genes (*Has1-3*) responsible for HA production [17], and our data indicated that KSHV infection prominently increased *Has1* transcripts, while having little or no effect on *Has2* or *Has3* transcripts (Fig. 1C). To confirm the role of *Has1* in KSHV-induced HA production, we directly targeted *Has1* by RNAi and showed that it significantly reduced HA production from KSHV-infected HUVEC (Fig. 1D).

Previous studies have shown that HA production is regulated by a multifunctional glycoprotein, CD147 (emmprin; basigin), in several types of tumor cells [12,18,19]. Our data confirmed that directly targeting CD147 by RNAi significantly reduced HA production from KSHV-infected HUVEC (Fig. 2A). Interestingly, targeting CD147 by RNAi also decreased *Has1* transcription but not *Has2* and *Has3* (Fig. 2B), while knock-down of *Has1* by RNAi decreased CD147 transcription but not *Has2* and *Has3* (Fig. S1), implying a positive feedback