נושא עבודת הגמר:

בעברית:

ההשפעה של נגיף הקפושי סרקומה והחלבון הלטנטי LANA-1 על הלוקליזציה והפרשת החלבון HMGB-1 בתאים מסוג B

באנגלית:

The influence of Kaposi's Sarcoma Associated Herpes Virus latency and LANA-1 protein on the localization and secretion of HMGB1 from B-cells

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האם הסטודנט התייעץ עם סטטיסטיקאי לצורך כתיבת הצעת עבודה זו? **לא.**

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המחלקה: הפקולטה לרפואה בגליל, אוניברסיטת בר אילן

The influence of Kaposis Sarcoma Herpes Virus latency LANA-1 protein on, High-mobility group box-1 protein, localization and secretion from B-cells.

1. Abstract

High-mobility group box-1 protein (HMGB1) acts as a typical 'alarmin', involved in the regulation of immune responses to infectious and non-infectious tissue damage. During normal cellular homeostasis, HMGB1 favors the nucleus, but with cell activation to tissue damaged, it is secreted through non-traditional pathways, actively and passively, which are not routed through the ER-Golgi. A recent study has found that HMGB1 interacts with the LANA-1 protein encoded by Kaposi's Sarcoma associated Herpes Virus (KSHV), which is a protein responsible for the latency phase in infected cells. Although it was previously described, still little is known about the active secretion of HMGB1 from B-cells and what exactly is the influence of the HMGB1-LANA interaction, on HMGB1 localization. Since the inflammasome (a known inducer of HMGB1 secretion) is activated in KSHV associated malignancies and in KSHV infected cells, it may emerge as a useful serological biomarker for diagnosis and screening of those malignancies.

Through this study we will assess, if and how the cellular localization and secretion of HMGB1 is influenced by the presence of the KSHV with emphasis to the LANA-1 protein, related to KSHV infected B-cell, and KSHV–associate malignancies such as in Primary Effusion Lymphoma (PEL).

Key words: HMGB1, KSHV, LANA.

2. Introduction

Although originally described already in 1973 as a highly conserved nuclear protein involved in DNA replication, transcription and repair[1], high-mobility group box-1 protein (HMGB1) has emerged as a key mediator in the regulation of immune responses to infectious and non-infectious tissue damage, by exhibiting all the properties of a typical ‘alarmin’.

On average, HMGB1 is found at concentrations of 10^6 molecules per cell and non-specifically binds to the minor grooves in DNA. The nuclear localization of HMGB1 and its affinity for DNA is regulated through phosphorylation and acetylation, and has been found to have a dynamic relationship with chromatin[2]. There are two known pathways for the release of HMGB1 into the extracellular milieu - passive and active. The passive pathway is attributable to cell death from damage or necrosis relevant to almost all cell types, and the active pathway is secretion from immune cells activated by pro-inflammatory stimuli. It is also well known that immune cells such as monocytes or dendritic cells, can secrete actively the HMGB1 protein as a response to both exogenous and endogenous signals, each in which a different mechanism[3]. Vettermann et al [4] also showed an active secretion from B-cells. Active secretion of HMGB1 is generally through non-traditional, leaderless pathways which are not routed through the ER-Golgi[5]. During normal cellular homeostasis

the dynamic relationship of HMGB1 with the nucleus and cytoplasm favors the nucleus. However, HMGB1 localizes in secretory lysosomes when, for instance, hyper-acetylated on lysine residues and then is released upon appropriate signaling stimuli. When HMGB1 is not acetylated, it remains localized to the nucleus and is not secreted or released[6].

The inflammasomes, a large caspase-1-activating protein complexes, were recently shown to play a critical role in mediating the extracellular release of HMGB1 from activated and infected immune cells[7]. Singh et al, demonstrated that endothelial telomerase-immortalized human umbilical cells (TIVE) supporting KSHV stable latency and PEL (cavity-based B-cell lymphoma 1 [BCBL-1]) cells show evidence of inflammasome activation, such as the activation of caspase-1 and cleavage of pro-IL-1β and pro-IL-18[8].

Many viruses encode inhibitors for Casp1 or the inflammasome. This suggests that the inflammasome plays a crucial role in promoting anti-viral immunity. Not surprisingly, many viruses are detected by the inflammasome, thus, viruses make a concerted effort to avoid inflammasome detection.[9] During the lytic phase KSHV encodes Orf63[10], which inhibits the NLRP1/3 , an essential component of inflammasome.

Recently, Shamay et al. have found[11] that HMGB1 interacts with the LANA-1 protein from the Kaposi's Sarcoma Herpes Virus (KSHV), which is a major factor among other proteins, responsible for the latency phase in infected B-cells[12]. Although recent evidences had demonstrated the interactions between HMGB1 and lytic phase proteins in the cycle of the KSHV, such as Replication Transcriptional Activator (RTA), *the implication of the interaction between HMGB1 and latency proteins such as LANA-1*, which can have a substantial and fundamental effect on the eruption and progression of the malignancies caused by the KSHV, *is not completely understood*.

3. Specific aims - Our research hypothesis is that the localization and release of HMGB1 from cells is influenced by the presence of the viral LANA-1 protein and/or KSHV infection.

(1) We will test how the concentrations of the total HMGB1 released from the cells and how the localization of the HMGB1 inside the cells changes between an infected B-cells and non-infected cells.
(2) We will determine the changes in secretion and localization between cells that are in the latency phase and those in the lytic phase.
(3) As mention before, LANA-1 may be the key protein but is not solely responsible for the latency phase of the KSHV. There are several proteins involved in maintaining the latency phase among LANA-1, such as vCYC and vFLIP[13]. Given that, it is much necessary to certify that the changes in HMGB1 secretion is indeed influenced by LANA-1 and HMGB1 interaction, as we will demonstrate further on.
(4) Additionally, we will test the possibility that KSHV inhibits the active HMGB1 secretion from an infected cell by adding a known inducer of HMGB1 secretion to an infected and non-infected B-cell using exogenous and endogenous stimulus mimicking onset activation of infectious and non-infectious tissue damage respectively.

4. Significance of study

As already mentioned, HMGB1 might be involved in the development and progression of several malignancies, and emerges as a potential diagnostic marker for cancer. Serum HMGB1 could be a useful serological biomarker for diagnosis and screening of those malignancies as well as for their prognosis.[14,15]

Furthermore, there is little known about the implication of HMGB1-LANA interaction and its effect on malignancies caused by the KSHV. By better understanding of the mechanism in which HMGB1, a major pro-inflammatory and late mediator regenerative tissue repair factor[16], functions, it is possible to implicate from this knowledge and use it to influence its eruption and progression and to develop a diagnostic tool as well as therapeutics to malignancies caused by the KSHV.

5. Material and methods

Cell culture

(1) BC3 and BCBL1 (Based Cavity B-cells Lymphoma) are primary effusion lymphoma, B-cells latently $KSHV^+$.
(2) BJAB are B-cell lymphoma not infected ($KSHV^-$) that will serve as a negative control.
(3) BJAB cells that were infected in-vitro with KSHV (serves as latently infected cells in the background of BJAB). These latently infected cells can be induced into lytic phase by anti-human IgM [17].

Sandwich ELISA for HMGB1 - To detect HMGB1 in cell culture medium samples, an ELISA will be performed. Using HMGB1 ELISA Kit (NeoBiolab, 96-well plate) by manufacture instruction. The absorbance at 405 nm will be measured using a microplate spectrophotometer. Concentrations of HMGB1 in the samples will be calculated using Softmax software.

Immunofluorescence assay - cells will be attached to chamber slides (LabTek) that were pre-treated with Poly-Lysine. Cells will be washed in PBS, fixed in 3% paraformaldehyde–PBS for 20 min at 25°C, washed in PBS, permeabilized in 0.2% Triton X-100–PBS for 10 min at 25°C, and washed again in PBS. All staining are performed at 37°C for 1 h. HMGB1 will be detected with a rabbit anti-HMGB1 antibody (Thermo Scientific Pierce Antibodies, A16617) and rhodamine - conjugated donkey anti-rabbit secondary antibody (Jackson) and DAPI (Enzo Life Sciences, Inc, ENZ-52404) for co-localization compression. To detect LANA's presence we will use an anti-LANA rat antibody and either a fluorescein isothiocyanate (FITC) - or rhodamine-conjugated donkey anti-rat IgG secondary antibody (Jackson). All antibodies will be used at a 1:200 dilution in 1% BSA–PBS.

Analysis of HMGB1 secretion by western blotting - cells will be seeded in 6-well cell culture plates, the culture supernatants will be centrifuged to remove cells. The concentrated supernatants will be subjected to SDS-PAGE under reducing conditions and transferred to a nitrocellulose membrane. The membrane will be placed in blocking solution for 1 h and incubated for an additional 1 h with the HRP-conjugated rabbit anti-HMGB1

antibody (Bioss Inc, bs-0664R-HRP). After washing with PBS containing 0.1% Tween 20, bound IgG was visualized by ECL Plus (GE Healthcare, Buckinghamshire, UK) and analyzed with Ez-Capture II (ATTO, Tokyo, Japan). The acquired images were analyzed with CS Analyzer 3.0 software (ATTO).

LANA Tet^{on} stable cells - pTRE-LANA were cloned by digestion of DY52 with BglII and ligation into the pTRE2pur BamHI site. In order to generate BJAB-TRE and BJAB-LANA, BJAB Tet-On cells were transfected with pTRE2pur and pTRE-LANA, respectively. Stable cells were selected with 200 ug/ml G418 and 2 ug/ml puromycin[11].

Immunoprecipitation - For immunoprecipitation assays cells will be lysed in 1 ml lysis buffer (50mMTris [pH 7.9], 100mM NaCl, 0.5 mM EDTA, 2% glycerol, and 0.2% NP-40 plus protease inhibitors [0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 □g/ml aprotinin, and 10 □g/ml leupeptin]), sonicated for 10 s, and cleared by centrifugation. Extracts will be pre cleared using protein-A/G PLUS-agarose (Santa Cruz Bio- technology, Inc.) and immunoprecipitated with anti-HMGB1 (Thermo Scientific Pierce Antibodies, A16617) on A/G PLUS-agarose. Beads will be washed 6 times with the same buffer, and bound proteins will be detected by Western blotting - in order to distinguish between phosphorylated and acetylated HMGB1, specific antibodies will be used for blotting. Anti-phosphoserine residue antibody will be used for phosphorylated-HMGB1 (Biorbyt, orb18260) and anti-acetylated-lysine residue antibody (BioLegend, 623402) will be used for acetylated-HMGB1.

Study design

Our experimental research is both descriptive and quantitative – we will describe any changes detected in the localization and secretion of HMGB1 with respect to the negative and positive control groups through all four questions. Also, we will measure the quantity of secreted HMGB1 by means of computerized technique as described in the method section.

(1) In order to test the possibility that HMGB1 cellular localization and secretion is modulated by KSHV infection, we will start by comparing infected and non-infected cells. All our experiments will be performed on cells grown in tissue culture. Using BC3 and BCBL1 as infected cells and BJAB that will serve as a negative control. We will also use BJAB cells that were infected in-vitro with KSHV (serves as latently infected cells in the background of BJAB). As a positive control we will use known inducers of HMGB1 secretion, such as LPS and ATP. We will first use an immunofluorescence assay (IFA) to demonstrate the localization of HMGB1 inside the cell with respect to the nucleus. Later on, cells will be grown and their growth medium will be collected and analyzed using sandwich ELISA, to demonstrate and calculate the secreted HMGB1 in cultures.
(2) We will demonstrate the changes in secretion and localization between BJAB-KSHV that are in the latency phase and those in the lytic phase, using same methods as previously described (sandwich ELISA and IFA).
(3) Next, as already mentioned, it is crucial to differentiate the LANA-HMGB1 interaction among other latency protein interaction with HMGB1. Doing so by using LANA Tet^{on}

plasmid. Meaning, we will use two BJAB cultures; one cell culture which had gone through stable LANA Tet^{on} plasmid integration and the second integrated an empty plasmid (negative control). We will then add doxycycline to induce the production of LANA protein and measuring the total HMGB1 medium concentration and localization inside the cell, using ELISA and IFA.

(4) Finally, we will induce B-cell activation by applying endogenous factor (TNFα) and exogenous factor (LPS), causing phosphorylation and acetylation of HMGB1 respectively[18]. We will then use immunoprecipitation to isolate the HMGB1 protein followed by Western blot with anti-phosphoserine and anti-acetylated-lysine antibodies to determine the presence of HMGB1 modification, in infected and un-infected cells.

Variables - the main variables as referred to each question:

(1) There are two variables in this case; the first variable is the localization of HMGB1 from the nucleus to the cytoplasm and extracellular matrix. The second variable is the amount of HMGB1 secreted.
(2) As mentioned above, only tested on BJAB-KSHV at lytic and latent phases.
(3) As mentioned above, only tested on BJAB, one with stable LANA-Tet^{on} plasmid and an empty plasmid.
(4) The main variable is the value concentrations of the two forms of HMGB1 secreted, with the first three measurements of it. This will gives us an idea about which mechanism of secretion is attributed to the KSHV infection.

The rest of experimental techniques and method as described above.

6. By the exclusive and rigorous supervision of Dr. Shamay the student will:

- Present a research question derived from a proposal of the research supervisor.
- Review relevant and up-to-date data about the research question.
- Design the relevant experiment.
- Carry out the experiment using material and methods as planed in advance.
- Collect and process the data.
- Draw conclusion.
- Write a final paper.

7. Time schedule –

Proposal approval > onset of research design - 5.7.2015

Onset of research design > collecting data – 23.8.2015

Collecting data > data analysis – 23.9.2015

Data analysis > Writing the paper – 11.10.2015

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