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Importin-β and exportin-5 are strong biomarkers of productive reoviral infection of cancer cells☆

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ABSTRACT

Acute reoviral infection has been extensively studied given the virus's propensity to target malignant cells and activate caspase-3 mediated apoptosis. Reovirus infection of malignant N1E-115 mouse neuroblastoma cells led to significant increased expression of importin-β and exportin-5 mRNAs (qRTPCR) and proteins (immunohistochemistry) which was partially blocked by small interfering LNA oligomers directed against the reoviral genome. Co-expression analysis showed that the N1E-115 cells that contained reoviral capsid protein had accumulated importin-β and exportin-5, as well as activated caspase 3. Reoviral oncolysis using a syngeneic mouse model of multiple myeloma similarly induced a significant increase in importin-β and exportin-5 proteins that were co-expressed with reoviral capsid protein and caspase-3. Apoptotic proteins (BAD, BIM, PUMA, NOXA, BAK, BAX) were increased with infection and co-localized with reoviral capsid protein. Surprisingly the anti-apoptotic MCL1 and bcl2 were also increased and co-localized with the capsid protein suggesting that it was the balance of pro-apoptotic molecules that correlated with activation of caspase-3. In summary, productive reoviral infection is strongly correlated with elevated importin-β and exportin-5 levels which may serve as biomarkers of the disease in clinical specimens.

1. Introduction

The nuclear core complex is an essential anatomic compartment between the nucleus and cytoplasm that regulates the passage of macromolecules either by diffusion or, for proteins > 40 kDa, through an energy dependent mechanism [1-3]. Cells may use nuclear trafficking to either repress or activate a constitutively expressed protein with p53 and NFK-B representing two well-documented examples [4–7]. The exportins and importins are the key proteins in regulating energy dependent nuclear trafficking. Importins have two subunits, α and β with importin-β capable of transporting molecules alone. Exportin-5 has been much studied for its ability to facilitate oncogenesis [8-12].

Reovirus is a double stranded RNA virus that is not associated with clinical disease in humans. Reovirus has been the subject of intense research given its strong propensity to selectively infect cancer cells [13-19]. The ability of reovirus to lyse cancer cells is highly correlated with productive infection as defined by robust capsid production and likely involves caspase-3 mediated apoptosis, immune mediated cytotoxic cancer cell death, and necroptosis, a receptor-interacting protein 3 kinase mediated process [13-18]. Reoviral mediated tumor cell lysis has been documented for a wide range of cancers including multiple myeloma. Reolysin has recently received fast track designation in metastatic breast cancer after a randomized phase 2 study revealed improvement in overall survival for reovirus treated patients [19].

Viruses can use importins and exportins to facilitate their infectivity [20-25]. The HIV-1 protein Rev. can bind to both importin-β and some exportins which plays an essential role in viral infection since, in the

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absence of Rev, late structural mRNAs of HIV-1 are unable to leave the nucleus with reduced infectivity [20,21]. The herpes simplex virus protein ICP27 likewise is required for nuclear export of viral mRNAs that is prerequisite for infectivity, but also competes and inhibits the binding of host nuclear traffic [22]; the same principle applies to vesicular stomatitis virus [23]. The obligate need of some RNA and DNA viruses to use host importin-β for nuclear trafficking has been exploited in anti-viral treatments. One such example is dengue fever where ivermectin has been shown to block nuclear transport of the viral NS5 protein that, in turn, markedly reduces viral infectivity [24,25].

Caspase-3 mediated cell death is a common pathway in which both cytoplasmic and nuclear concomitants have been detailed in previous works [8,9,26,27]. Nuclear transport of caspase-3 involving the importin-β system is necessary for caspase-mediated apoptosis [8,9]. Several members of the bcl2 family, which can regulate both caspase-3 activation and apoptosis, must traffic in and out of the nucleus to mediate cell death [26,27]. The purpose of this manuscript is to examine the effect of acute reoviral infection on importin-β and exportin-5 expression and to correlate this with caspase-3 mediated cell death and the expression of multiple bcl2 family members. This work will hopefully serve as the foundation to examine the role of importin-β and exportin-5 as general biomarkers of acute viral infection in the diagnostic pathology laboratory.

2. Materials and methods

2.1. Cell lines

The murine neuroblastoma cell line N1E -115 (ATCC CRL-2263) was obtained from the ATCC and grown according to recommended specifications. The cells were grown to 50% confluence and infected either with reovirus (4.5×10^{10} $TCID_{50}$) or sham infected. The cells were then grown for 48 h and either fixed in 10% neutral buffered formalin for in situ hybridization/immunohistochemistry or rapidly frozen at − 20C, placed in RNaLater, and RNA extracted for qRTPCR.

2.2. Syngeneic mouse myeloma model

Animal experiments were performed under the University of Calgary Institutional Animal Care and Use Committee approval (AC12-0126) and confirmed to all regulatory standards. We utilized the Vk*MYC syngeneic mouse model of myeloma to confirm our in vitro observations in an in vivo setting. This model has been described before [28-31]. C57BL/6 wild type (WT) non-irradiated mice were transplanted with Vk*MYC myeloma (Vk12598, a kind gift from Dr. L. Bergsagel) and monoclonal paraproteins (M- spike) were assessed on a weekly basis. Following the appearance of an M –spike approximately 3 weeks post transplantation of tumor, the mice in 6 groups were treated as follows. 1) vehicle control (carboxy methyl cellulose (Sigma-Aldrich, Ontario, Canada) + tween 80), 2) live reovirus (5×10^8 PFU), 3) dead reovirus (5×10^8 PFU), 4) bortezomib (Selleckchem, Ontario, Canada) (1 mg/kg), 5) live virus (5×10^8 PFU) + bortezomib (1 mg/kg), 6) dead virus (5×10^8 PFU) + bortezomib (1 mg/kg). Mice were sacrificed after 4 days of treatment and their spleens were formalin fixed and paraffin embedded.

2.3. qRTPCR

RNA (425 ng) was retro-transcripted using the High Capacity cDNA Reverse Transcription kit (Life Technologies Waltham, MA) according to the manufacturer's protocol. The comparative real-time PCR was performed in triplicate, including no-template controls and analyzed using QuantStudio 12 K Flex Real-Time PCR System. The Ct Average of each triplicate was used to perform the relative quantification analysis. RNA input was normalized using mouse GAPDH as the reference gene and the relative expression was calculated using the comparative Ct method.

2.4. In situ hybridization

Reoviral RNA in situ hybridization was done with a previously published protocol that utilizes a 5′ digoxigenin tagged set of two LNA oligoprobes as well as a detection system that uses an antibody against digoxigenin that is conjugated to alkaline phosphatase [15,16]. The chromogens nitro-blue tetrazolium and 5-bromo-4-chloro-3′-indolyphosphate yields a blue signal with nuclear fast red as the counterstain. The sequence of the LNA modified oligomers directed against a conserved region of reovirus serotype 3 Dearing strain at nt 3203-3222 and 3057-3076, respectively was as follows: TGCGCAAGAGGCAGCA-ATCG and TTCGCGGGCCTCGCACATTC.

2.5. Immunohistochemistry

Our immunohistochemistry protocol has been previously published [15,16]. Each sample was tested for the following antigens: activated caspase-3, bcl2, BAD, BIM, p53, reoviral protein μ1 capsid protein (confirmed by Western blot analysis of the reoviral proteins expressed in the N1E infected cells, data not shown) importin-β, exportin-5 (ABCAM, Cambridge MA), BAX, BAK, Mcl1, PUMA, NOXA (Enzo Life Sciences,Farmingdale, NY) and the anti- type 3 Dearing reoviral antibody that can detect the λ, μ and σ capsid proteins (a gift from Oncolytics Biotech Inc., Calgary, Canada). The analyses were done on the automated Leica Bond platform with the modification that we used the Enzo Life Sciences peroxidase anti-mouse/rabbit conjugate (catalogue # ADI-950-113-0100) as this reduced background.

2.6. Co-expression analysis

Co-expression analyses were done using the Nuance system (CRI) as previously published [15,32]. In brief, a given tissue was tested for two different antigens using fast red as the chromogen for one target followed by immunohistochemistry using DAB (brown) as the second chromogen with hematoxylin as the counterstain. The results were then analyzed by the Nuance and InForm systems in which each chromogenic signal is separated, converted to a fluorescence based signal, then mixed to determine what percentage of cells were expressing the two proteins of interest.

2.7. Statistical analyses

Statistical analysis was done using the InStat Statistical Analysis Software (version 3.36) and a paired *t*-test (also referred to as a "repeated measure t-test") testing the null hypothesis that the probability of the expression of a protein in the virus treated cells/tissues was equivalent to the probability in the non-viral associated samples. The null hypothesis was rejected if the significance level was below 5%.

3. Results

3.1. Reovirus and N1E 115 cells

The murine neuroblastoma cell line N1E 115 form large cohesive groups with many interconnected dendritic cell processes in culture. Within 24 h of exposure to live type 3 Dearing reovirus, the cells became less cohesive with a marked reduction in their interdigitating processes (Fig. 1). These cell pattern changes were minimized if the live reovirus was added at the same time as two LNA modified oligomers directed against the highly conserved sequence of the double stranded RNA virus (Fig. 1).

Immunohistochemistry documented that the N1E-115 cells expressed RAS (data not shown) and, thus, are sensitive to reoviral infection. Forty-eight hours after exposure the cells were tested for reoviral RNA and capsid viral protein and scored in a blinded fashion. The results are provided in Table 1A and 1B. Note that reoviral RNA was