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KSHV downregulation of galectin-3 in Kaposi's sarcoma

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Galectins are a family of proteins that share an affinity for beta-galactoside containing glycoconjugates. In prostate, ovarian and breast cancer, downregulation of galectin-3 is associated with malignancy and tumor progression. Kaposi's sarcoma (KS) is characterized as an angioproliferative tumor of vascular endothelial cells and produces rare B cell lymphoproliferative diseases in the form of primary effusion lymphomas and some forms of multicentric Castleman's disease. Kaposi's sarcoma-associated herpesvirus (KSHV) is the etiological agent of KS. We found reduced levels of galectin-3 expression in a significant fraction of latency-associated nuclear antigen (LANA)-positive spindle cell regions in human archival KS tissue and as measured in KS tissue microarrays. Here we demonstrate that galectin-3 protein expression is downregulated 10-fold in 10-day KSHV-infected dermal microvascular endothelial cells (DMVEC) accompanied by downregulation of message. There is loss of galectin-3 staining in KSHV-infected DMVEC by dual labeled immunohistochemistry in LANA-positive spindle cells. We observed a consistent downregulation of galectin-3 by time-course transcriptional analysis. Of the galectins assayed, only galectin-1 was also downregulated in KSHV-infected DMVEC. We examined 86 KS tumors; 19 were LANA positive (22%) and 67 LANA negative (78%). All 86 tumors were found to be galectin-3 positive; 11 of 19 showed reduced expression of galectin-3 in LANA-positive spindle cell regions. Our data suggest that KSHV vFLIP and LANA are the viral genes targeting galectin-3 downregulation. The contribution of host factors to the pathogenesis of KS is essential for early detection and development of innovative therapies for treatment.

Keywords: endothelial cells/galectin-3/immunohistochemistry/Kaposi's sarcoma/transcriptional suppression

Introduction

By organization and number of conserved carbohydrate recognition domains (CRDs), galectins can be subdivided into three main groups: the prototype group (galectins-1, -2, -5, -7, -10, -11, -13, -14 and -15 with one CRD), the chimera group (galectin-3, with one CRD and an extended N-terminal domain) and the tandem repeat group (galectins-4, -6, -8, -9 and -12) having two homologous CRDs as well as a unique linker sequence (Hirabayashi and Kasai 1993; Barondes, Castronovo, et al. 1994; Barondes, Cooper, et al. 1994).

Galectin-3 is the most studied and has been implicated in a number of biological functions including cell proliferation, cell cycle regulation, angiogenesis, cell adhesion, cell differentiation, anti-apoptotic activity, RNA processing and tumorigenesis (Dagher et al. 1995; Inohara et al. 1998; Perillo et al. 1998; Hughes 2001; Dumic et al. 2006). Tumor progression associated with upregulation of galectin-3 includes that observed in colorectal, thyroid and hepatocellular carcinomas, human angiosarcomas, stomach cancer, glioblastomas and head and neck cancer (Irimura et al. 1991; Lotan et al. 1994; Schoeppner et al. 1995; Xu et al. 1995; Bresalier et al. 1997; Fernandez et al. 1997; Hsu et al. 1999; Johnson et al. 2007; Saussez et al. 2007). Tumor progression associated with downregulation of galectin-3 includes cancers of the colon, breast, ovaries, uterus, prostate, tongue, skin and liver fluke-associated cholangiocarcinomas (Castronovo et al. 1992, 1996; Lotz et al. 1993; van den Brule et al. 1994, 1996; Honjo et al. 2000; Pacis et al. 2000; Mollenhauer et al. 2003; Junking et al. 2008).

Galectin-3 is expressed by primary human umbilical vein endothelial cells along with galectins-1 -8, and -9, with marginal expression of galectins-2, -4 and -12 (Thijssen et al. 2008). No transcriptional spliced variants of galectin-3 were observed. Galectin-3 is found in human endothelial cells from kidney, placenta and colon but not from liver (Thijssen et al. 2008). It is widely expressed in human angiosarcomas from skin, bone scalp, breast, ileum and liver, which is another type of aggressive endothelial cell tumor (Johnson et al. 2007). There is also evidence that galectin-3 shuttles from the nucleus to the cytoplasm (Moutsatsos et al. 1986; Califice et al. 2004) and that this differential compartmentalization of galectin-3 may be associated with different biological functions (Davidson et al. 2002).

Kaposi's sarcoma-associated herpesvirus, also known as KSHV or human herpesvirus type 8, is the etiological agent of Kaposi's sarcoma (KS) (Chang et al. 1994; Moore and Chang 1998, 2002). KS is the most common malignancy in HIV patients who have no access to anti-retroviral drugs; hence, it is the most common AIDS-associated malignancy found in southern Africa. As the prevalence of KS in HIV/AIDS is directly related to the patient's state of immune suppression, KS is also a problem in transplant recipients who require immunosuppressive drugs for allograft acceptance (Alkan et al. 1997; Mbulaiteye and Engels 2006). KS is characterized as an angioproliferative tumor, and KSHV produces rare B-cell lymphoproliferative diseases in the form of pleural effusion lymphomas and some forms of multicentric Castleman's disease (Cesarman et al. 1995; Soulier et al. 1995; Cesarman and Knowles 1997). KSHV encodes approximately 90 genes; however, in the latent phase of virus replication in vivo, only a few genes are expressed and are referred to as

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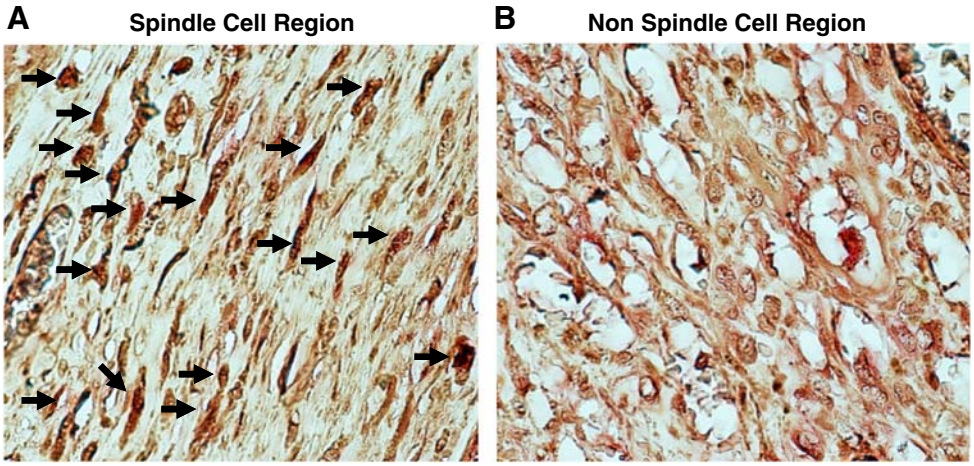

Fig. 1. Galectin-3 expression in KS tumor tissue. Briefly, paraffin-embedded KS tissue was deparafinized in xylene and hydrated in graded alcohol, and antigen unmasking was performed in citrate buffer in a microwave. Cells were stained by dual labeled IHC for KSHV LANA (Vector Red, red) and galectin-3 (DAB, brown). No counterstain was used in this procedure. Brightfield images were photographed on a NIKON TE2000S microscope at total magnification of ×600. (**A**) A spindle cell region, (**B**) a non-spindle cell region observed in a representative KS tissue sample. Arrows in (**A**) point to spindle cell containing LANA nuclear staining (red), while the diffuse galectin-3 staining (brown) seen in (**B**) is lacking.

latency genes (Jenner and Boshoff 2002). Major KSHV genes expressed during latency include the latency-associated nuclear antigen (LANA), viral FLICE inhibitory protein (vFLIP) and viral cyclin D (vCYC-D) (Jenner and Boshoff 2002). KS tumor cells are primarily latently infected, and in vivo analysis of archival KS tissue demonstrates that only a small fraction of cells are supporting virus lytic cycle replication at any given time (Staskus et al. 1997; Chiou et al. 2002).

LANA, a viral protein expressed during latency, has been shown to dysregulate tumor suppressor pathways associated with p53 and Rb (Friborg et al. 1999; Radkov et al. 2000), thereby contributing to tumor progression. It has been shown to interact with H-*ras* to transform primary rat fibroblasts and associates with GSK-3 beta-altering Wnt signaling pathways, resulting in the accumulation of beta-catenin. The accumulated beta-catenin upregulates Tcf/Lef regulated genes (Fujimuro et al. 2003). Most recently, LANA has been shown to inhibit transforming growth factor (TGF)-beta signaling through silencing of the TGF-beta type II receptor (Di Bartolo et al. 2008).

KSHV vFLIP has been reported to both block Fas-mediated (Bertin et al. 1997; Thome et al. 1997) apoptosis and activate NF–kB pathways by associating with IkB kinase (IKK) (Chaudhary et al. 1999; Liu et al. 2002). Moreover, vFLIP binds IKK-gamma, leading to activation of IKK (Field et al. 2003). vFLIP is required for spindle cell formation and contributes to the inflammation cascade seen in KS (Chiou et al. 2002).

vCYC-D has been shown to phosphorylate the Rb protein and inactivate Bcl2 which would inhibit apoptosis and allow the cell to bypass cell cycle checkpoints (Horenstein et al. 1997; Ojala et al. 2000). These KSHV latency proteins dysregulate the described molecular pathways and contribute to tumorigenesis. Combined with paracrine effects of angioproliferative viral genes (Liu et al. 2001), cellular factors must play an important role in the establishment of KS.

To our knowledge, there have been no previous reports of galectin-3 dysregulation in Kaposi's sarcoma. We demonstrate downregulation of galectin-3 expression in KSHV-infected dermal microvascular endothelial cells (DMVEC), the same cells that undergo conversion to spindle cells mimicking those found in KS tumors replicating KSHV (Boshoff, et al. 1995; Renne et al. 1998; Ciufo et al. 2001; Lagunoff, et al. 2002). We also report reduced expression of galectin-3 in archival KS tissue in LANA-positive spindle cell compared to non-spindle cell regions. Moreover, we observed downregulation of galectin-3 transcription and show that vFLIP and LANA are the viral genes that likely target galectin-3 downregulation.

Results

Galectin-3 expression in human archival KS tissue

Dysregulation of human galectins have long been associated with malignancies. Differential expression of the galectin family of proteins have been associated with several tumor types that include head and neck cancer, colon cancer, adenocarcinoma, prostate cancer and tumors of the central nervous system.

Galectin-3 dysregulation has been implicated in several tumors with the notion that dysregulation of galectin-3 could play an important role in tumor progression and metastasis. To our knowledge, there are no reports of galectin-3 expression analysis in KS tissue or reports of galectin-3 involvement in KS pathogenesis. Therefore, archival KS tissue from a nodular lesion that represents the most advance stage of KS disease (marked by increased levels of virus replication and spindle cells) was used in this study. These spindle cells represent the main cell type found in the nodular or advanced form of KS.

Using dual labeled immunohistochemical staining of archival KS tissue, we showed reduced expression of galectin-3 in spindle cell areas of tumor tissue defined as having greater than 70% of LANA-positive nuclei when using Vector Red as a substrate for alkaline phosphatase (Figure 1). Galectin-3 expression is shown as brown staining of the cytoplasm using 3,3-diamino-benzidine (DAB) as a substrate for horseradish peroxidase

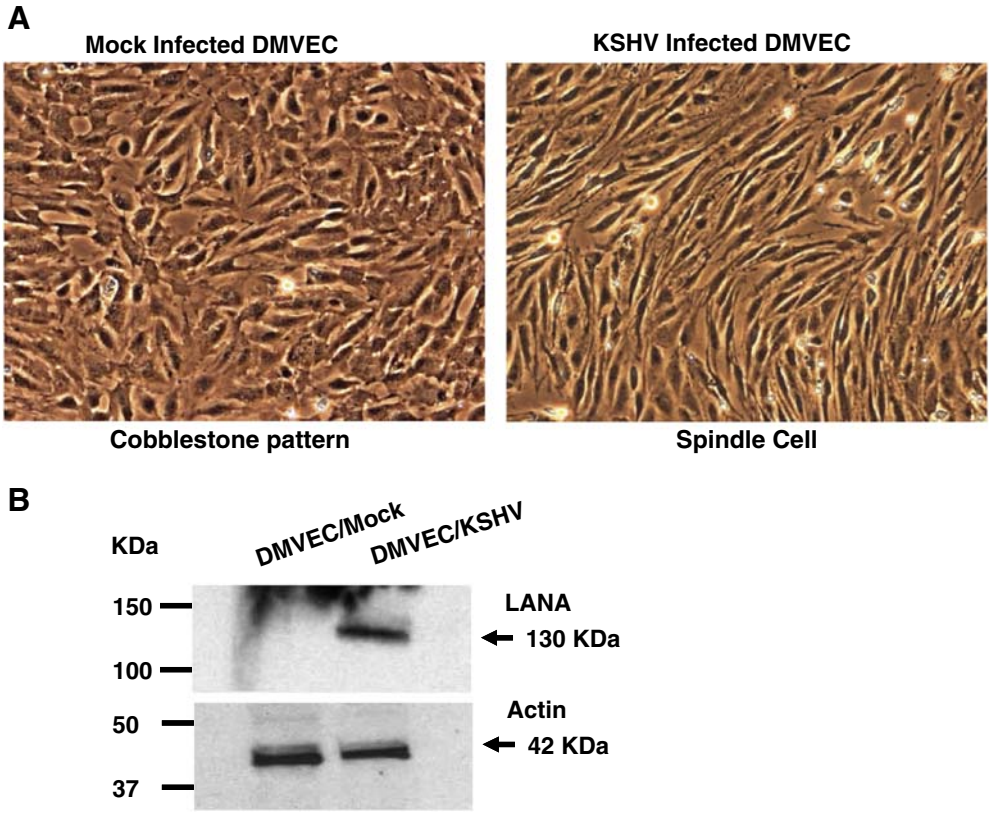

Fig. 2. Results using human dermal microvascular endothelial cells (DMVEC) that were either mock infected or infected with KSHV for 10 days. Cells were infected with BCBL-1 virus at a moi 0.01 (or mock infected) and cultivated in EBM-2 complete media. Phase images were taken with a NIKON TE 2000S microscope mounted with a CCD camera. (**A**) Mock infected cells with a cobblestone-like morphology at left and KSHV-infected cells with the characteristic spindle-shape morphology at right. (**B**) Western blot analyses indicating that KSHV-infected cells strongly express LANA in stark contrast to mock infected cells. For Western blots, lysates (15 μg) from mock and KSHV-infected DMVEC were electrophoresed in 4–20% PAGE gels, transferred to nitrocellulose membranes and screened for expression of KSHV LANA using a mouse anti-human LANA antibody (Novacastra) at a 1:2000 dilution. Protein bands show evidence of ~130 kDa band in lysates from KSHV-infected DMVEC when compared to the mock infected DMVEC.

(Figure 1). Galectin-3 is more highly expressed in non-spindle cell areas of the tumor, whereas in spindle cell areas of the tumor, galectin-3 expression is reduced (Figure 1). LANA-positive spindle cells with red nuclear staining are shown by arrows (Figure 1). This observation prompted us to examine galectin-3 expression in KSHV-infected DMVEC cells.

KSHV infection of DMVEC and galectin-3 expression by immunofluorescence

We infected DMVEC with body cavity based lymphoma (BCBL)-1 virus and observed morphological changes over time that are consistent with the spindle cell phenotype of KSHV-infected DMVEC (Ciufo et al. 2001). The mock infected cells displayed a cobblestone morphology consistent with normal early passaged DMVEC (Figure 2A). Spindle-shaped cells expressed LANA that was not expressed in mock infected cells (Figure 2B). Dual labeled immunofluorescence of KSHV-infected spindle cell populations stained for LANA, which displayed a nuclear punctate staining pattern in infected cells (Figure 3A). In many tumors, galectin-3 has been found in the cytoplasm; however, it has also been seen in the nucleus (Califice et al. 2004). Cytoplasmic galectin-3 expression in tumor cells has been associated with anti-apoptotic effects. Our in vitro results show expression of galectin-3 to be cytoplasmic with some nuclear staining exclusively in uninfected DMVEC (negative for LANA staining; Figure 3A). Comparing these results with a microscopic examination of 60–100 optical fields with 60–80% of DMVEC in culture expressing LANA led us to believe that KSHV infection specifically downregulates galectin-3 expression. We could not find galectin-3 expression in the cytoplasm or nucleus of any LANA-positive DMVEC (Figure 3A). This would suggest that galectin-3 is undergoing transcriptional suppression or protein degradation during KSHV infection.

We also observed downregulation of galectin-3 protein expression by Western blot. Densitometry analysis revealed a 10-fold reduction in galectin-3 protein expression in KSHV-infected DMVEC when compared to mock infected controls (Figure 3B). This downregulation in KSHV-infected cells is statistically significant with a standard deviation of 0.85 and a p value of 0.000104. Of interest is a previous observation by Hsu et al. (2000), that macrophages from galectin-3 knockout mice (gal3−/−) exhibit a spindle-shaped morphology. Galectin-3 downregulation by KSHV could potentially contribute to the spindle cell phenotype observed in KS tissue and in vitro KSHV-infected DMVEC.

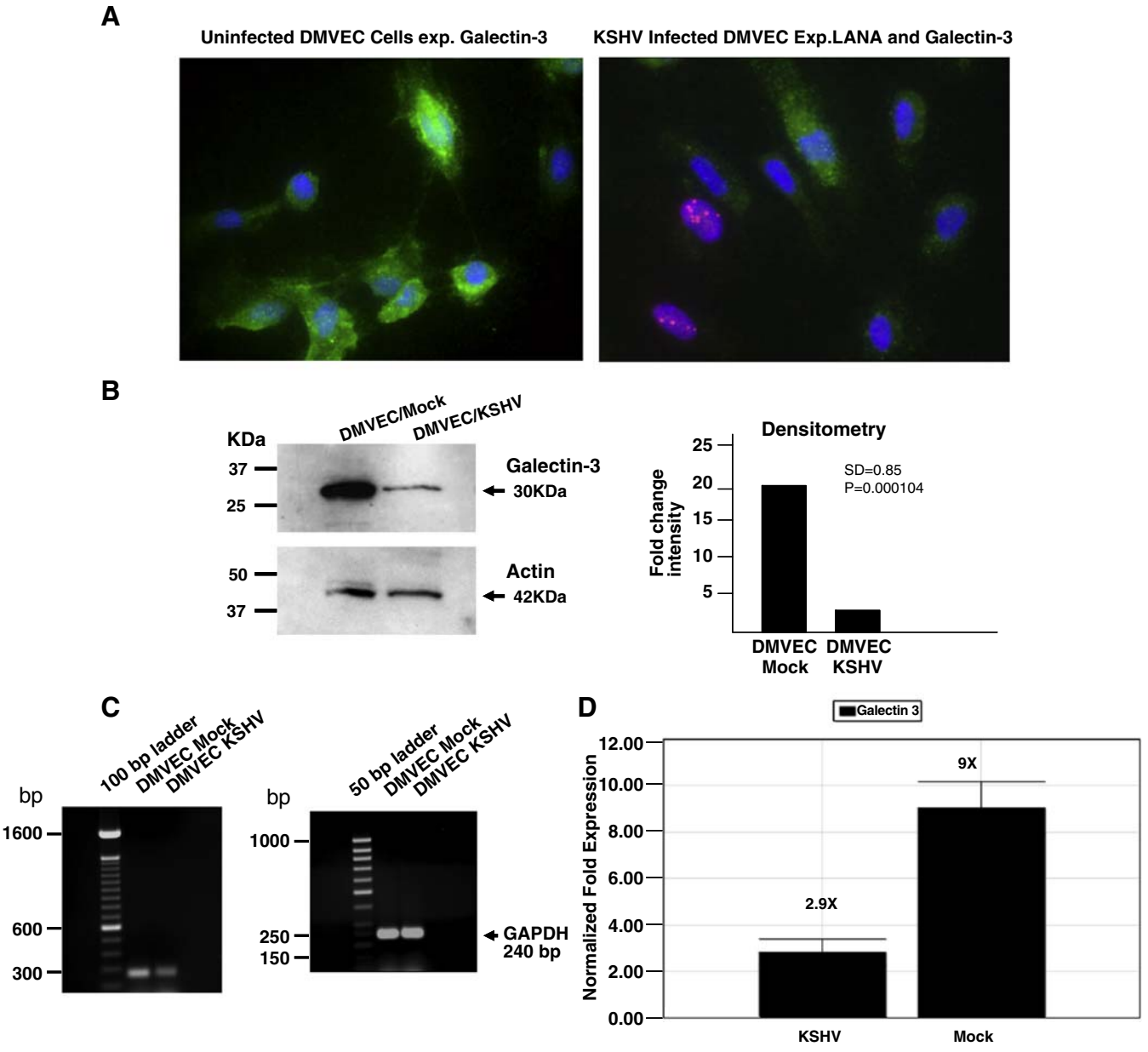

Fig. 3. Galectin-3 expression in KSHV and mock treated cells at 10 days. (**A**) Dual labeled immunofluorescent staining of KSHV-infected and mock infected DMVEC. DMVEC were mock infected or infected with BCBL-1 virus at a moi 0.01 and cultivated in EBM-2 complete media. Uninfected DMVEC in chamber slides seeded at 2×10^5 cells/well were stained by IFA using a polyclonal goat antibody to human galectin-3 (R&D Systems, AF1154) at a 1:100 dilution. Infected cells were dual labeled with a monoclonal antibody to KSHV LANA (LANA/ORF73, Vector Laboratory VP-H913) and a goat polyclonal antibody to galectin-3, both at 1:50 dilution, then labeled with donkey anti-mouse rhodamine tagged secondary antibody together with a rabbit anti-goat FITC tagged antibody as a mixture. Photographs were taken with a NIKON TE 2000S microscope at a total magnification of ×600. Shown in (**A**) are galectin-3 positive cells (green) with cytoplasmic and some nuclear staining. DAPI was used to stain nuclei. In (**A**), LANA-positive cells that were rhodamine stained and have a nuclear punctate pattern; galectin-3 positive cells were stained green with FITC. (**B**) Western blot analysis of galectin-3 protein expression in KSHV-infected DMVEC. Mock and KSHV-infected DMVEC lysates (15 μg) were electrophoresed in 4–20% PAGE gels, transferred to nitrocellulose membranes and then screened for expression of galectin-3 using a goat anti-human galectin-3 antibody (R&D Systems) at a 1:2000 dilution. Proteins bands of ~30 kDa showed much reduced levels of galectin-3 in lysates from KSHV-infected DMVEC, compared to the mock infected controls. Densitometry analysis revealed a 10-fold decrease in the amount of galectin-3 protein in DMVEC lysates from KSHV-infected cells. (**C**) RT-PCR analysis of galectin-3 mRNA expression in KSHV-infected DMVEC. Ten nanograms of cDNA from mock and KSHV-infected DMVEC were amplified by PCR using galectin-3 gene-specific primers in Exon 3. PCR products were electrophoresed in 1.5% agarose, and PCR DNA fragments were sized using a 100-bp ladder. The expected fragment size for galectin-3 is 256 bp. GAPDH was amplified as a loading control with a fragment size of 240 bp. (**D**) Real-time qPCR analysis of galectin-3 showing a 3.1-fold decrease in mRNA in KSHV-infected DMVEC cells relative to mock infected controls.

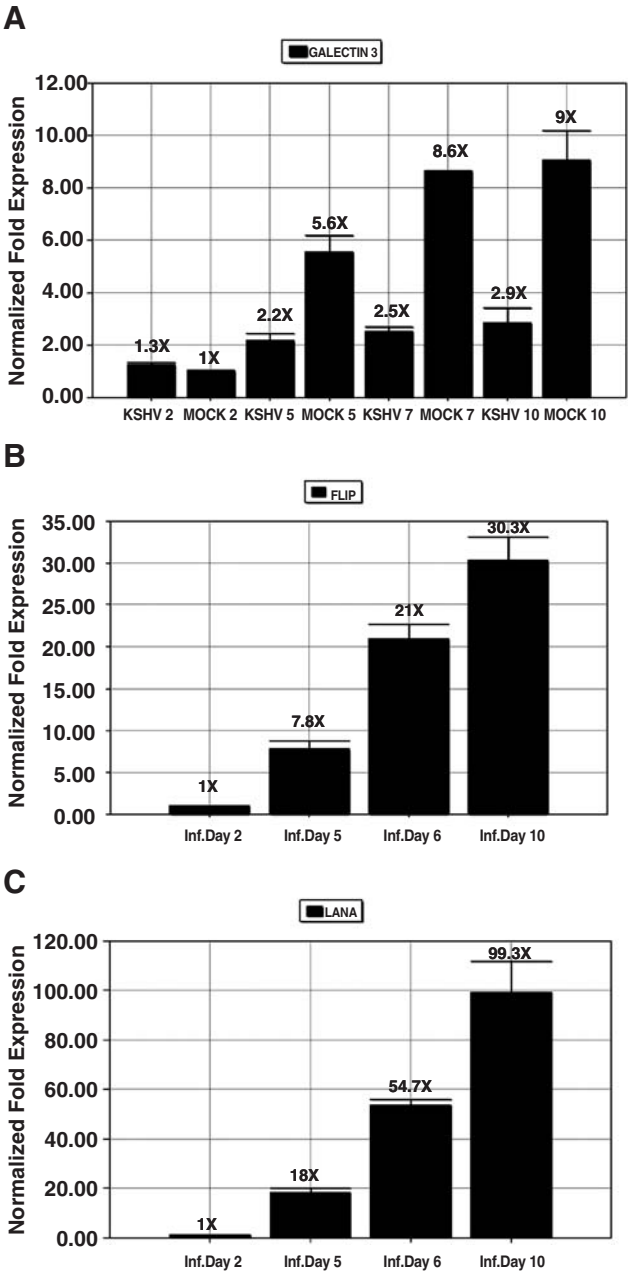

Fig. 4. (**A**) The temporal expression of galectin-3 by qRT-PCR in KSHV-infected DMVEC at 2, 5, 7 and 10 days post infection compared to mock infected controls cells. (**B**) Temporal expression of vFLIP by qRT-PCR 10 days post infection compared to mock infected control cells. (**C**) Temporal expression of KSHV LANA 10 days post infection compared to mock infected control cells. All values for KSHV-infected DMVEC cells were normalized to GAPDH.

Transcriptional downregulation of galectin-3 in DMVEC after KSHV infection

Galectin-3 expression is modulated during serum starvation and is upregulated by serum in cultured cells (Moutsatsos et al. 1986). Galectin-3 is classified as an immediate early gene, and changes in galectin-3 expression are transcriptional. To determine if galectin-3 mRNA expression level is downregulated in KSHV-infected DMVEC, we performed reverse transcriptase polymerase chain reaction (RT-PCR) with RNA from infected DMVEC and mock infected control cells. By RT-PCR, we find transcriptional suppression of galectin-3 message in DMVEC after infection compared to mock infected controls (Figure 3C).This result supports our protein expression data that galectin-3 transcription is downregulated by KSHV infection. We have quantified these data by quantitative real-time RT-PCR which shows a 3.1-fold decrease in galectin-3 transcription in KSHV-infected DMVEC cells compared to mock infected control cells (Figure 3D). The difference in the downregulation of galectin-3 protein expression and the downregulation of galectin-3 transcription suggests that some type of post transcriptional downregulation of galectin-3 expression occurs in KSHV-infected DMVEC cells.

Galectin-3 turnover in KSHV-infected DMVEC

To examine the turnover of galectin-3 in KSHV-infected DMVEC, we performed a time course at 2, 5, 7 and 10 days post infection. We observed largely a consistent downregulation of galectin-3 over time by quantitative real-time RT-PCR (Figure 4A). At 2 days post infection, galectin-3 is slightly upregulated compared to controls, but at 5, 7 and 10 days in our transcriptional assay we show galectin-3 downregulated in KSHV-infected cells compared to mock infected control cells (Figure 4A). All values for KSHV-infected DMVEC cells were normalized to glyceraldehyde phosphate dehydrogenase (GAPDH).

Next we wanted to determine if there was a correlation between downregulation of galectin-3 and increased transcription of vFLIP and LANA over time in KSHV-infected DMVEC. We performed a time-course analysis for vFLIP and LANA expression in KSHV-infected DMVEC by qRT-PCR. We find that there is direct correlation in time between increased vFLIP and LANA transcription with transcriptional downregulation of galectin-3 in KSHV-infected DMVEC cells compared to mock infected controls (Figure 4B and C). Mock infected cells showed no demonstrable vFLIP or LANA expression over this same period of time.

To verify galectin-3 dysregulation in other endothelial cell populations, we performed transcriptional analysis of mock and KSHV-infected human umbilical cord vein endothelial cells (HUVEC). RT-PCR analysis revealed a slight increase in galectin-3 expression in HUVEC after KSHV infection (10 days) in comparison to mock infected control cells (Figure 5B). This suggests that galectin-3 expression after KSHV infection can vary among different endothelial cell populations. This observation is supported by a study performed by Lotan et al. (1994), who observed different galectin-3 expression levels in endothelial cells isolated from different tissues.

Transcriptional analysis of other galectins expressed in DMVEC after KSHV infection

There are 15 galectins reported to date, but only 11 are expressed in humans. We examined, in KSHV-infected DMVEC, changes in the transcriptional profile of other members of the galectin family that have been reported to be associated with malignancies. Using gene-specific oligonucleotide primers pairs (Table 1), we examined galectins-1, -4, -7, -8 and -9 because of their associations with malignancies. Galectins-2, -6, -10, -12, -13 and -14 have no current associations

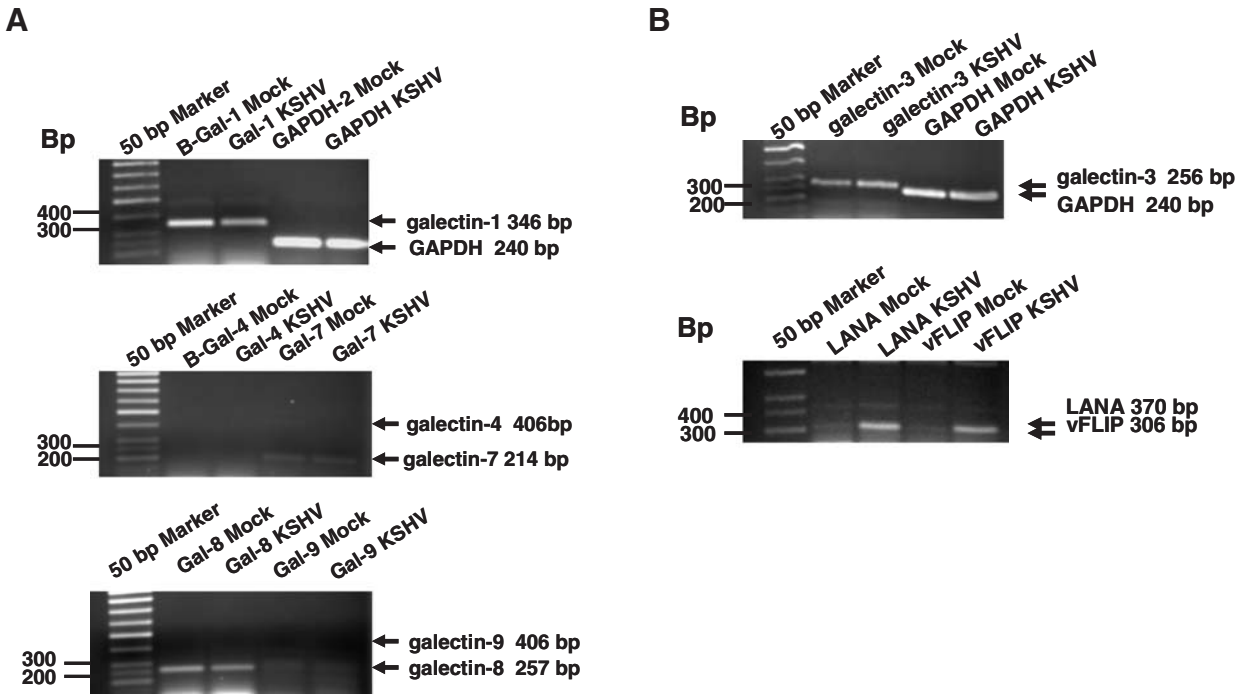

Fig. 5. Transcriptional analysis of other galectins implicated in malignancy after KSHV infection of DMVEC cells, compared to controls. (**A**) Amplification of galectin-1 at 10 days (346 bp) using GAPDH as a control; galectin-4 levels (not expressed) and an equal but low level expression of galectin-7 in both KSHV-infected and control cells; equal levels of galectin-8 and no expression of galectin-9 mRNA in both KSHV-infected and mock infected DMVEC under the conditions of assay. (**B**) Galectin-3 transcriptional expression in HUVEC mock or KSHV infected with viral infection markers amplified for vLANA and vFLIP.

with cancer and were not included in this study. We found galectin-1 mRNA expression to be downregulated in KSHV-infected cells (Figure 5A). We find no change in mRNA expression levels of galectins-7 and -8 after KSHV infection of DMVEC for 10 days (Figure 5A). Using our conditions for RT-PCR, we find that galectins-4 and -9 are not expressed in either mock or KSHV-infected DMVEC at detectable levels (Figure 5A).

Analysis of KS tissue microarrays

Tissue microarrays (TMA) were initially immunohistochemistry (IHC) stained with LANA only to determine positive tumor cores, then a replicate serial sectioned array was dual stained for LANA and galectin-3 by immunohistochemistry and analyzed by brightfield microscopy. A counterstain was not included to avoid discrepancies between substrate colors. LANA was stained with DAB, and galectin-3 was stained with the Vector Red substrate for alkaline phosphatase. LANA-positive cells in the tumors were visualized by an intense brown nuclear punctuate staining pattern (Figure 6). Stained tissue cores were observed at ×200 magnification. IHC results from the TMA revealed that there were 126 KS tumor cores from different patients spotted on the array. We found 86 intact tumors on the array with approximately 40 tumor cores destroyed or missing due to tissue processing. We identified 19 (22%) LANA-positive KS tumors and 67 (78%) LANA negative tumors.

Table 1. Human galectin primer sequences for RT-PCR and qRT-PCR

Gene	Size of product (bp)	Primer	Sequence (5′–3′)
Galectin-1	346	Forward	TGGTCGCCAGCAACCTGAATCTCA
		Reverse	TAGTTGATGGCCTCCAGGTTGAGG
Galectin-2	305	Forward	AAGATCACAGGCAGCATCGCCGAT
		Reverse	CTTACGCTCAGGTAGCTCAGGTGG
Galectin-3	256	Forward	GATGCGTTATCTGGGTCTG
		Reverse	GCACTTGGCTGTCCAGAAGATG
Galectin-4	406	Forward	CACATGAAGCGGTTCTTCGTGAAC
		Reverse	TCAGCTGTTGATGGCAATGTCCGG
Galectin-5	214	Forward	TCCCAATGCCAGCAGGTTCCATGT
		Reverse	GAAGCCGTCGTCTGACGCGATGAT
Galectin-6	257	Forward	TCCAGGTGGATCTGCAGAATGGCA
		Reverse	GATCCTGTGGCCATAGAGCAGAGT
Galectin-7	483	Forward	CAGGCACCCATGGCTCAAACTAC
		Reverse	TATCAGACTCGGTAACGGGGGT

Human galectin RT-PCR and qRT-PCR oligonucleotide primer pairs for galectin family members implicated in malignancies.

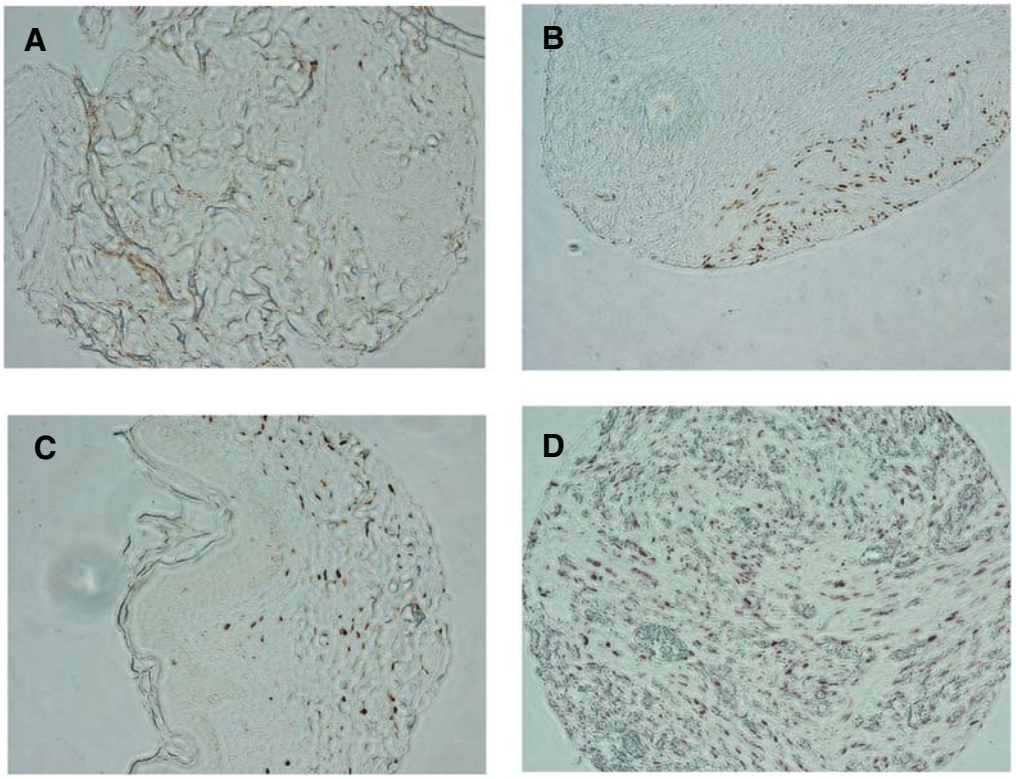

Fig. 6. Immunohistochemistry of human KS tissue microarrays. Single-label IHC on tissue microarrays for LANA was performed with no counterstain. Here we show variability among tumor specimens from different patients with low to high viral burden. (**A**) Low viral burden with few spindle cells. (**B**) Focal infection with localized spindle cells. (**C**) Increased viral burden and spindle cells. (**D**) High viral burden with an increased number of spindle cells.

All 86 tumors were found to be galectin-3 positive, and 11 of 19 tumors showed reduced expression of galectin-3 in LANA-positive spindle cell regions (Table 2). The finding of 11/19 KS tumors that were LANA positive resulting in the downregulation of galectin-3 is statistically significant when compared to LANA-negative tumor specimens. The statistical significance we speak of (11/19 tumors) relates to the observation that, of the 19 LANA-positive tumors, 11 had a reduced galactin-3 level compared to 0 of 67 LANA-negative tumor cores, all of which had significant expression of galactin-3.

We conclude that examination of more tumors is warranted. It should be possible to measure tissue galectin-3 amounts directly by real-time PCR in the future upon acquisition of more KS tumor specimens with negative matched control tissue.

Effects of KSHV latency genes on HeLa cell expression of galectin-3

HeLa cells transfected with KSHV latency genes LANA, vFLIP, vCYC-D and two KSHV immediate early genes, vMIR1 and vMIR2, were analyzed for downregulated galectin-3 expression by Western blot. These genes represent the subset of viral genes expressed by KS tumors in vivo (Jenner and Boshoff 2002). The KSHV immediate early genes vMIR1 and vMIR2 are included in this study because of their association with degradation of major histocompatibility complex class I (MHC class I) molecules on the surface of antigen presenting cells. Both vMIR1 and vMIR2 have been demonstrated to have ubiquitin-protein ligase activity that directs MHC class I molecules to lysosomes for degradation (Ishido et al. 2000; Coscoy and Ganem 2001; Means et al. 2002; Dodd et al. 2004). Other proteins found to be downregulated by vMIR1 and vMIR2 include B7-2, CD31/PECAM-1 by K5, and ICAM-1. Most recently, vMIR1 and vMIR2 have been shown to downregulate gamma interferon receptor-1 (Li et al. 2006; Mansouri et al. 2006).

Mock transfected control cells and cells transfected with pBluescript plasmid DNA yielded similar levels of galectin-3 protein expression as HeLa cells transfected with vMIR1,

Table 2. Analysis of KS tissue microarrays

Tumor cores spotted on TMA	Intact tumor cores	Total LANA-positive cores	LANA-negative tumor cores	Galectin-3-positive cores	Tumor cores showing reduced expression of galectin-3 in LANA spindle cell regions
126 100%	86 68%	19 22%	67 78%	126 All examined	11/19 58%

Intact tumor cores, tumor cores examined that were not destroyed by processing during mounting, fixation or staining procedure.

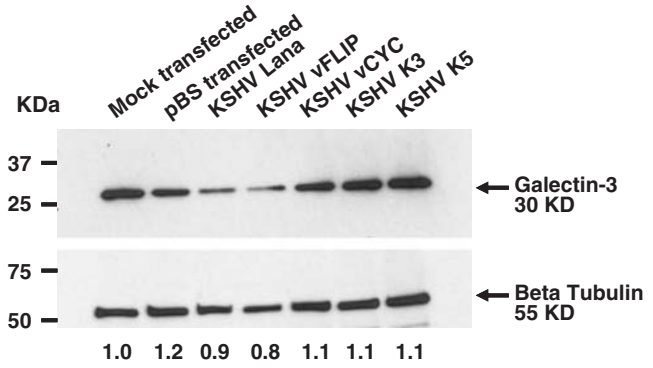

Fig. 7. Effects of KSHV latency genes on galectin-3 expression in HeLa cells. HeLa cells in six-well plates at a density of 5×10^5 were transfected with 2 μg of LANA, vFLIP, vCyc-D, K3 or K5. Lysates were made in RIPA buffer 48 h post transfection, and galectin-3 expression levels were analyzed by Western blot. HeLa cells mock transfected and HeLa cells transfected with pBluescript plasmid DNA were used as controls. Note the downregulation of galectin-3 after vFLIP transfection and LANA transfection. The respective ratios of galectin-3 to beta-tubulin are given under each lane.

vMIR2 and vCYC-D (Figure 7). However, second to KSHV LANA, KSHV-vFLIP transfection showed the greatest reduction in galectin-3 expression (Figure 7). Reduction of galectin-3 levels by vFLIP is intriguing because vFLIP is required for spindle cell formation in DMVEC (Grossmann et al. 2006; Ciufo et al. 2001; Hayward 2003), and macrophages from galectin-3 knockout mice have been shown to exhibit spindle cell formation (Hsu et al. 2000). This suggests that the reduction of galectin-3 by vFLIP induces spindle cell formation; however, 48 h after transfecting HeLa cells with vFLIP, we saw no significant change in galectin-3 expression or cell morphology by RT-PCR and dual labeled immunofluorescent staining (data not shown). However, while immunofluorescent analysis of galectin-3 transfected HeLa cell showed no demonstrable change in galectin-3, we did observe a significant decrease in galectin-3 expression by vFLIP and LANA in immunoblot analysis of whole cell lysate (Figure 7).We have performed densitometry analysis on the immunoblot and have determined the ratios of galectin-3 to beta-tubulin, and these values appear under each lane (Figure 7). We propose that, because HeLa cells are transformed, vFLIP may induce spindle cell formation in HeLa cells by a different mechanism than that observed in DMVEC cells. The role of vLANA in suppression of galectin-3 remains unclear.

Discussion

Galectin-3 has been implicated in tumor progression and metastasis (Danguy et al. 2002). It is also known that in prostate, ovarian and breast cancers and in cancer of the tongue, downregulation of galectin-3 is associated with malignancy (van den Brule et al. 2004). Infected DMVECs in culture show characteristic spindle cell morphology, In these infected cells, we found a 10-fold reduction in galectin-3 protein expression levels by immunoblot analysis in comparison to mock infected controls. We also observed reduced expression of galectin-3 staining by immunofluorescence assay (IFA) and by dual labeled immunohistochemistry in LANA-positive spindle cells in nodular archival KS tissue from a KS patient. Nodular KS tumors have been identified as the most advance stage of KS disease. Based on the exclusion of galectin-3 from both the nucleus and cytoplasm of infected DMVEC, the cells that support KSHV infection in KS tissue, galectin-3 levels may vary in the early-stage KS lesion referred to as the patch/plaque form of KS compared to the nodular form (Jenner and Boshoff 2002; Simonart et al. 2000).

We also observed transcriptional suppression of galectin-3 message by RT-PCR in KSHV-infected DMVEC compared to mock infected controls. By time-course transcriptional analysis of galectin-3 expression in KSHV-infected and uninfected DMVEC cells, we find consistent downregulation of galectin-3 with 2 days post infection which shows a marginal increase in galectin-3 expression. Downregulation of galectin-3 overtime correlates with increased transcriptional expression of KSHV vFLIP and LANA.

We examined other galectin family members with our transcriptional assay which included galectins-1, -4, -7, -8, and -9 because of their associations with malignancies. With the exception of galectins-1 and -3, which showed downregulation of mRNA expression in KSHV-infected DMVEC at 10 days, all other galectins in this study were either unchanged (galectins-7 and -8) or undetectable (galectins-4 and -9) in both mock and KSHV-infected DMVEC.

In contrast, HUVEC cells infected with KSHV showed a slight increase in galectin-3 expression at 10 days of incubation compared to controls which may reflect differences in cellular microenvironments and/or the effects of replication kinetics of KSHV in HUVEC (Figure 5B).Observations by Thijssen et al. (2008) noted that basal endothelial galectin-3 in vivo expression differs in human vascular endothelial cells isolated from different sites, possibly due to their micro-environment. In vitro activation responses also appear to be galectin specific.

We found by Western blot that HeLa cells express high levels of galectin-3, and transfection of HeLa cells with LANA, vFLIP, vCYC-D, vMIR1 and vMIR2 expression plasmids revealed that KSHV vFLIP and KSHV LANA are the most likely genes involved in the suppression of galectin-3 protein expression. No effect on galectin-3 expression was observed by vMIR1 or vMIR2 transfection. Grossmann et al. have shown that vFLIP is required for spindle cell conversion of DMVECs from their normal cuboidal shape during KSHV infection in vitro. This DMVEC conversion greatly resembles the spindle-like morphology of KS tumor cells. It is also known that spindle cell formation of macrophages is observed in galectin-3 knockout mice; therefore, one proposed mechanism for vFLIP spindle cell conversion could include downregulation of galectin-3. However, after transient transfection of HeLa cells with a vFLIP expression plasmid, we found no change in galectin-3 expression by immunofluorescence or changes in cell morphology in these cells (data not shown).

Spindle cell formation by KSHV-infected DMVECs, which are primary cells, requires some time to develop in culture. Spindle cell formation in HeLa cells, which are transformed cells, may require high sustained levels of vFLIP expression before galectin-3 expression levels are altered sufficiently and morphological changes in cells can be observed. Therefore, it may be difficult to compare galectin-3 levels in vFLIP transfected cells with galectin-3 levels in viral infected cells. DMVECs, as primary cells, are not very amenable to transfection and would require

the development of adenovirus or lentiviral vectors to transduce vFLIP with high efficiency into DMVEC and determine its effects on galectin-3 expression and cell morphology.

Tissue microarrays reveal that a significant number of LANA-positive KS tumors show a reduction in galectin-3 expression when compared to LANA negative tumors. We identified 19 (22%) LANA-positive KS tumors and 67 (78%) LANA negative tumors. All 86 tumors were found to be galectin-3 positive, and 11 of 19 tumors showed reduced expression of galectin-3 in LANA-positive spindle cell regions (Table 2).

We believe that the 11 tumors that show reduced expression of galectin-3 is a conservative number because several of the remaining eight specimens were found to be inconclusive. This was due to variations in staining profiles among these tumors.

We acknowledge that vFLIP can stimulate expression of NFKb (Guasparri et al. 2004), and NFKb-like binding sites are found in the galectin-3 promoter. However, regulation of the galectin-3 promoter via NFKb transcription has been shown to involve the apoptosis-associated protein nucling (Lui et al. 2004). Mechanisms that regulate galectin-3 expression are complex and will require further investigation.

Galectin-3 is known to interact with acetyl lactosamine residues of glycoconjugates, such as laminin, and alters the expression of laminin binding protein receptor at the surface of cancer cells. This contributes to the pathological association between laminin and tumor cells (van den Brule et al. 1996). Downregulation of galectin-3 could facilitate laminin–cancer cell interactions which results in a more invasive and metastatic tumor phenotype. Altered expression of galectin-3 in cancer cells is associated with the acquisition and progression of the malignant phenotype. Malignancy will vary among different tumors in relation to overall levels of galectin-3 expression as well as with the presence and absence of nuclear and cytoplasmic localizations of galectin-3 in cancer cells.

Materials and methods

Cells and viruses

The BCBL-1 cell line originally isolated from a body cavity based lymphoma was cultured in RPMI 1640 media (Gibco, Grand Island, NY) until the cell density reached 3×10^6 cells/mL, then lytic cycle virus replication was induced with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) at 20 ng/mL and sodium butyrate at 0.3 ng/mL. Twenty-four hours post induction, cells were washed twice in phosphate-buffered saline (PBS) to remove butyrate, and induction was continued with TPA for 5 days. Cell-free virus was isolated and concentrated by differential centrifugation. DMVEC maintained in complete EMB-2 media (Lonza, Basel, Switzerland) at passage level 4 were infected at a multiplicity of infection (moi) of 0.01, and mock infected cells were used as controls. Ten days post infection, cells were prepared for immunofluorescent staining. HeLa cells were maintained in Dulbecco's modified essential medium supplemented with 10% fetal calf serum and penicillin/streptomycin.

Immunofluorescence

Chamber slides containing both confluent infected and uninfected DMVEC were washed twice with PBS pH 7.4, air dried, and fixed in absolute methanol for 10 min at −20°C. Cells were then air dried again for 15 min, hydrated in Tris saline (0.05 M Tris pH 7.4) for 5 min and then incubated for 1 h with a mixture of monoclonal antibodies to KSHV LANA at a 1:50 dilution (Vector Laboratories, Burlingame, CA) plus a goat polyclonal antibody to galectin-3 (R&D Systems, Minneapolis, MN) at a 1:100 dilution. The cells were then washed three times with Tris saline and incubated for 30 min with a mixture of a secondary donkey anti- mouse IgG antibody conjugated with rhodamine red-X and donkey anti-goat antibody conjugated to fluorescein isothiocyanate (FITC) (Jackson ImmunoResearch, West Grove, PA) both at a 1:100 dilution in PBS. The cells were later washed three times in Tris saline and mounted with Vectashield mounting media (Vector Laboratories) containing 1.5 μg/mL of 4′,6-diamidino-2-phenylindole (DAPI). Fluorescence was observed and images photographed with a Nikon TE 2000S fluorescent microscope mounted with a charge-coupled device (CCD) camera.

Western blot analysis

To determine levels of galectin-3 protein expressed by DMVEC before and after KSHV infection, cell extracts from infected and mock infected cells were prepared using RIPA lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA pH 8.0, 1% NP40, 0.5% deoxycholate sodium, 0.1% SDS and proteinase inhibitor). Lysates were placed on ice for 30 min then clarified by centrifugation. Total protein was measured by bicinchoninic acid assay (Pierce). Fifteen micrograms of protein lysates from mock and infected paired samples were fractionated in 4–20% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, transferred to nitrocellulose membranes, blocked with 5% milk, 0.1% Tris-buffered saline Tween-20 (TBST) (0.1% Tween 20, 20 mM Tris, 150 mM NaCl) and incubated at 4°C overnight with either a polyclonal goat antibody to galectin-3 or a KSHV LANA monoclonal antibody at a 1:2000 dilution. Membranes were washed five times in washing buffer (0.1% TBST) and incubated for 1 h followed by incubation with a secondary antibody donkey anti-goat peroxidase conjugate (Santa Cruz Biotech, Santa Cruz, CA), at a dilution of 1:10,000. Immunoreactive bands were detected with SuperSignal West Dura Extended Substrate (Pierce) following exposure to X-ray film.

Densitometry

Densitometry analysis was performed on the immunoblot results (see Figure 3A) from mock and KSHV-infected DMVEC using a BIO RAD Chemi-Doc XRS gel docking system using Quantity One 4.6.2 software.

Immunohistochemistry

Dual labeled immunohistochemistry for galectin-3 and KSHV LANA was performed on archival AIDS-KS tissue fixed in formalin, embedded in paraffin, sectioned and placed on slides prior to immunostaining. KS biopsy specimens were procured according to the Institutional Review Board (IRB) approval policies of Johns Hopkins University School of Medicine. For immunostaining, antigen retrieval was performed in a microwave oven by pretreatment (500 W) in 50 mM citrate buffer at 95°C for 15 min. Cells were then incubated 30 min in PBS pH 7.2, containing 10% normal goat serum with 1% Tween-20

for blocking of nonspecific binding and permeabilization. Cells were then incubated with a goat polyclonal antibody to human galectin-3 (R&D Systems, AF1154) diluted 1:100 in blocking buffer. After 90 min of incubation, cells were washed and then incubated with a biotinylated rabbit anti-goat IgG antibody (DAKO) at a 1:100 dilution followed by a horseradish peroxidase–avidin conjugate (DAKO) at 1:500 dilution. Color development was achieved by incubating cells with the substrate DAB (SIGMA). The second labeling was achieved by an additional antigen retrieval step in the microwave as described above plus a second immunostaining with a monoclonal antibody to the KSHV LANA (Vector Laboratories). Cells were subsequently washed and then incubated with a biotinylated goat anti-mouse/rabbit IgG antibody (DAKO) at a 1:100 dilution followed by an alkaline phosphatase–strepavidin conjugate (Vector Labs, Burlingame, CA) at 1:500 dilution. Color development was achieved by incubating cells with the substrate vector red using a Vector Red alkaline phosphate substrate kit (Vector Laboratories) according to the manufacturer's instructions. Images were taken with a Nikon TE-2000S microscope mounted with a CCD camera.

RT-PCR

Total RNA was extracted from KSHV-infected DMVEC and mock infected cells using a Qiagen RNeasy Mini KIT (Qiagen, Valencia, CA). The RNA was DNAase treated prior to elution on the column according to the manufacturer's recommendations. Messenger RNA in 1 μg of each sample was primed using oligo-dT and reverse transcribed with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA.). Gene-specific primer pairs for all galectins assayed in this study and for KSHV vFLIP are shown in Table 1 and used 10–100 ng of cDNA for RT-PCR amplification. PCR was performed using PuReTaq Ready-To-Go PCR beads (GE Health Care, Buckinghamshire, UK) according to the manufacturer's recommendations. PCR was carried out in a MJ Mini thermocycler in a final volume of 25 μL. The cycling protocol used was 95°C for 5 min, 55°C for 30 s and 72°C for 1 min for 36 cycles, with a final extension at 72°C for 10 min. PCR products were electrophoresed in 1.5% agarose and DNA bands visualized by staining with ethidium bromide. Galectin-3 specific bands are shown as a 256-bp fragment. Primers for KSHV LANA amplification are designated as forward primer 5′ TACGGTTGGCGAAGTCACATC3′ and reverse primer 5′ CCTCGCAGCAGACTACACCTCCAC3′, and primers for KSHV vFLIP amplification are designated forward 5′ CGTCTACGTGGAGAACAGTGAGCT3′ and reverse 5′ CTGGGCACGGATGACAGGGAAGTG3′, which yielded fragments of 370 and 306 bp, respectively. GAPDH was amplified in mock and infected cells as a loading and quality control and is represented by a 240-bp fragment.

Real-time qPCR

Real-time PCR was performed in 96-well optical plates (Sorenson Bioscience, Inc.) using cDNA and a MyiQ Single Color Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) in 25 μl reaction volumes. A master mix was made according to manufacturer's instructions using SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) and forward and reverse primers at a concentration of 250 nM per well, made in RNAase DNAase-free H_2O. Primer sequences for quantitative real-time RT-PCR (qPCR) for galectin-3 are shown in Table 1; primer sequences for KSHV vFLIP and KSHV LANA were the same as used in our RT-PCR amplifications. The cDNAs from mock infected and KSHV-infected DMVEC cells were diluted 1:3 using RNAase DNAase-free H_2O; 3 μl of this dilution was added to each well. Control wells substituted water for cDNA. The cycling sequence included 95°C for 3 min, 95°C for 15 s, 60°C for 1 min, 95°C for 1 min, 55°C for 1 min and 55°C for 30 s for 81 cycles total. A GAPDH primer set shown in Table 1 was amplified and included for normalization. Data analysis was done with the Bio-Rad iQ5 Optical System Software Version 2.

Transfections

HeLa cells plated in six-well dishes at a density of 5×10^5 cells per well were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with 2 μg/well of plasmid DNA in serum-free media of LANA, vFLIP, vCYC-D, K3 (vMIR1) and K5 (vMIR2). After 18 h incubation at 37°C, DNA complexes were removed and replaced with fresh media containing serum. Cells were cultured for an additional 24 h. RIPA buffer lysates were prepared 48 h post transfection. Mock transfected cells and cells transfected with pBluescript plasmid DNA were used as controls.

Tissue microarrays

Tissue microarrays were obtained from the AIDS Cancer Specimen Resource Consortium (San Francisco, CA). All specimens were procured according to the IRB policies of Meharry Medical College. Paraffin-embedded KS tissue of 0.6 mm core sizes representing patch/plaque and nodular forms of KS from multiple patients were placed on slides. KS tumor tissue from the mouth, skin, tongue, soft plate and neck mass were included on the array. All tissues are from HIV-positive patients.

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Conflict of interest statement

None declared.

Abbreviations

BCBL, body cavity based lymphoma; CRDs, carbohydrate recognition domains; DAB, 3,3-diamino-benzidine; DAPI, 4′,6-diamidino-2-phenylindole; DMVEC, dermal microvascular endothelial cells; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde phosphate dehydrogenase; HRP, horseradish peroxidase; HUVEC, human umbilical cord vein endothelial cells; IFA, immunofluorescence assay; IHC, immunohistochemistry; IKK, IkB kinase; KS, Kaposi's sarcoma; KSHV, Kaposi's sarcoma-associated herpesvirus; LANA, latent associated nuclear antigen; MHC class I, major histocompatibility complex class I; moi, multiplicity of infection; PBS, phosphate buffer saline; PCR, polymerase chain reaction; qPCR, quantitative real-time RT-PCR; RT-PCR, reverse transcription polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TGF, transforming growth factor; TMA, tissue microarrays; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; vCYC-D, viral cyclin D homolog; vFLIP, viral Flice protein.

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