Galectin-3 expressed on different lung compartments promotes organ specific metastasis by facilitating arrest, extravasation and organ colonization via high affinity ligands on melanoma cells

Manohar C. Dange · Nithya Srinivasan · Shyam K. More · Sanjay M. Bane · Archana Upadhyya · Arvind D. Ingle · Rajiv P. Gude · Rabindranath Mukhopadhyaya · Rajiv D. Kalraiya

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Abstract Interactions between molecules on the surface of tumor cells and those on the target organ endothelium play an important role in their arrest in an organ. Galectin-3 on the lung endothelium and high affinity ligands poly-N-acetyllactosamine (polyLacNAc) on N-oligosaccharides on melanoma cells facilitate such interactions. However, to extravasate and colonize an organ the cells must stabilize these interactions by spreading to retract endothelium, degrade exposed basement membrane (BM) and move into parenchyma and proliferate. Here, we show that galectin-3 is expressed on all the major compartments of the lungs and participates in not just promoting adhesion but also in spreading. We for the first time demonstrate that both soluble and immobilized galectin-3 induce secretion of MMP-9 required to breach vascular BM. Further, we show that immobilized galectin-3 is used as traction for the movement of cells. Downregulation of galactosyltransferases-I and -V resulted in significant loss in expression of polyLacNAc and thus reduced binding of galectin-3. This was accompanied with a loss in adhesion, spreading, MMP-9 secretion and motility of the cells on galectin-3 and thus their metastasis to lungs. Metastasis could also be inhibited by blocking surface polyLacNAc by pre-incubating cells with truncated galectin-3 (which lacked oligomerization domain) or by feeding mice with modified citrus pectin in drinking water. Overall, these results unequivocally show that polyLacNAc on melanoma cells and galectin-3 on the lungs play a critical role in arrest and extravasation of cells in the lungs and strategies that target these interactions inhibit lung metastasis.

Keywords Organ specific metastasis · Lungs · Galectin-3 · Extravasation · Poly-N-acetyllactosamine · β1,6 branched N-oligosaccharides

Introduction

In spite of being the major cause of mortality in cancer patients; the underlying molecular mechanisms of metastasis are still poorly understood possibly due to the complexity of this multistep process [1]. To metastasize, tumor cells must break free from the primary site, create space for their movement, get into and survive in circulation [2]. Once in circulation, they are able to reach almost all organ sites. However, some metastasize in the anatomic vicinity, while others bypass several organs and colonize very specific organ sites [3]. The patterns of circulation and mechanical factors appear to dictate the regional spread [4]. However, organ specific metastasis is believed to be facilitated by specific interactions between the molecules on the tumor cells and the target organ, growth environment and chemotactic factors released from the target organ [3, 5–7].
Vascular endothelium is the first barrier that a tumor cell must overcome to colonize an organ. Organ endothelium also provides specific receptors/ligands for organ specific homing of cancer cells [8, 9]. Extravasation of leucocytes at the inflamed site has provided useful clues to the overall process of extravasation which involves rolling, adhesion and extravasation [10]. Selectins and their ligands promote rolling and retard the movement of leucocytes. Firm endothelial adhesion is facilitated by activated integrins and their counter receptors. This is followed by diapedesis which involves endothelial retraction, degradation of vascular basement membrane (BM) and movement into organ parenchyma [10, 11].

Tumor cells are also believed to utilize similar mechanisms for extravasation and each of these steps could be rate limiting [12, 13]. The vascular endothelium has been shown to express specific set of surface molecules on different organs [14]. Tumors reportedly adhere preferentially to the endothelial cells or the ‘outside out’ endothelial cell membrane vesicles, derived from their metastatic site [15]. VE-cadherin, integrins, Ig class of cell adhesion molecules, selectins, carbohydrates and their lectin receptors are among the major class of molecules on the endothelial cells and on the cancer cells, which are believed to aid adhesion of cancer cells to the target organ [7, 16–19]. Constitutive expression of E-selectin on vessels of the bones or on inflamed organ endothelium has been shown to facilitate organ homing of cells expressing specific E-selectin ligands [10, 20].

However, the participation of E-selectins and its ligand in promoting metastasis in the lungs appears remote. Extravasation occurs predominantly in the micro-vascular capillaries in pulmonary circulation, which are too small to allow rolling [21, 22]. Lung colonizing cancer cells have been shown to get redirected to liver upon forced expression of ligands for E-selectin on hepatic cells [23]. Other receptors/ligands implicated in lung specific interactions include dipeptidyl peptidase IV (DPP-IV), Lu-ECAM1, VCAM-1, CLCA2 on the lung endothelium and their counter receptors like fibronectin; CXCR4, β4 integrin on tumor cells [24–26]. Galectin-3 on the organ endothelium has also been implicated in promoting organ homing [27, 28]. It is a nucleo-cytoplasmic β-galactoside specific lectin that gets secreted out in a non classical manner and gets incorporated onto the cell surface and as part of the matrix and BM [29]. In mice, lungs were shown to express highest amounts of galectin-3 and express it constitutively on its vascular endothelium [27]. Several reports implicate T/Tn antigens on tumor cells in mediating both homophilic interactions and heterophilic interactions with endothelial cells via galectin-3 [19, 30, 31]. Apart from these interactions, galectin-3 in the host may also facilitate melanoma metastasis by modulating immune response, in particular innate antitumor immunity [32, 33].

Using low and high metastatic variants of B16 melanoma cells, previous work by our group has shown that polyLacNAc substituted β1,6 branched N-oligosaccharides on cancer cells may serve as very high affinity, easily accessible form of ligands for galectin-3 [27, 28]. Galectin-3 shows >200-fold higher affinity towards polyLacNAc as compared to T/Tn antigens [34]. Galectin-3 on the lung microvascular endothelium appeared to promote lung metastasis by serving as an anchor to arrest circulating tumor cells carrying polyLacNAc substituted β1,6 branched N-oligosaccharides [27, 28]. Under flow conditions galectin-3 has been shown to bind to the glycoproteins carrying its ligands with high affinity as compared to the selectins to their ligands (Kd of 1 vs. 100–300 μM for selectins and is comparable to the interactions mediated by integrins) [35, 36]. However, just adhesion to vascular endothelium is not enough to establish metastatic foci.

The tumor cells need to displace endothelium, interact with and degrade the exposed vascular BM, move into organ parenchyma and proliferate within for effective metastasis [5, 7]. This was elegantly demonstrated by monitoring adhesive interactions with organ microvasculature and invasion by intra-vital microscopy of colon cancer cell lines differing in their metastatic potential. Although, adhesion occurred in micro-vasculatures of metastatic target organ only, their migration into organ parenchyma correlated with metastatic potential [12].

In the present communication, we demonstrate that galectin-3 present on all the major compartment of the lungs participates not just in promoting adhesion to vascular endothelium but also in all the subsequent events of extravasation. Further, we show that polyLacNAc substituted N- and not O-oligosaccharides participate in all these processes. Inhibition of expression of polyLacNAc or competitive inhibition of their interaction with the host galectin-3 both inhibited all these processes and thus metastasis.

Materials and methods

Reagents

TRIzol and Superscript TM amplification system for RT-PCR and Calcein AM were from Invitrogen, USA. Anti-mouse galectin-3 rat antibody was from R&D Biosystems, USA, and anti-Rat HRPO, anti-Goat HRPO from Santa Cruz Biotechnology, USA. E. coli BL 21 with pET3C plasmid containing a full-length human galectin-3 was a kind gift from Prof. Hakon Leffler, Lund University, Sweden. Biotinylated lectin Lycopersicon esculentum lectin (LEA), avidin–peroxidase, and streptavidin–FITC, were either from Sigma Chemical Company, USA or Vector
Labs, USA. Power SYBR Green PCR Master Mix was from Applied Biosystems. Anti-MMP-9 antibody, Primers for RT-PCR, Primers for real time PCR and for shRNA amplification, Phalloidin TRITC, Phalloidin FITC, DAPI, Pectin from citrus peel, Polybrene were purchased from Sigma Chemical Company. Dulbecco modified essential medium (DMEM) and fetal bovine serum (FBS) was purchased from Gibco, Invitrogen. All other chemicals were purchased locally and were of analytical grade. For experimental metastasis assay, inbred strain of C57BL/6 mice was used.

Cell lines

B16F1(F1) and B16F10 (F10) murine melanoma cell lines [37] were obtained from National Centre for Cell Sciences, Pune, India. The cell lines were expanded and frozen aliquots were stored in liquid nitrogen. Each aliquot was used only up to five passages in vitro. The metastatic potential of F10 cells is maintained by culturing melanoma colonies on the lungs obtained by performing experimental metastasis assay in C57BL/6 mice. Cell lines were routinely characterized for (C57BL/6) mouse specific origin and mycoplasma free status as described in supplementary methods.

Immunohistochemical detection of galectin-3 in mouse lungs

Immunohistochemical staining for galectin-3 was performed on 3-μm paraffin embedded sections as described in [27]. Sections were stained with rat anti-mouse galectin-3 monoclonal antibody followed by anti-rat horse radish peroxidase (HRPO) conjugate and developed with diaminobenzidine containing H₂O₂ as the substrate. Instead of the primary antibody, the control lung sections were treated with rat IgG in the concentration similar to the primary antibody. The slides were later counter stained with hematoxylin.

Purification of recombinant human galectin-3

Galectin-3 was purified as described in [38].

Adhesion assays

For Adhesion assays, either calcein AM labeled or tritiated thymidine labeled melanoma cells were used and were performed in 96 well plates coated overnight with galectin-3 (50 μg/ml) as described previously [39]. For labeling with calcein, melanoma cells were incubated with DMEM medium containing 3 μg/ml calcein. Fluorescence was measured in 96 well plate reader from Berthold Mithras LB-940 machine (Excitation filter-485 nm and Emission filter-535 nm). The percentage adhesion was calculated by considering F10 cells bound to galectin-3 as 100%.

Cell spreading assay

Melanoma cells were harvested, washed free of serum and 0.5 million cells were seeded in serum free DMEM on the coverslips coated overnight with 50 μg/ml galectin-3 in serum free DMEM at 4 °C. The cells were incubated for 45 min in a CO₂ incubator. Coverslips treated with serum free DMEM only, served as control. Bound cells were fixed in 4 % paraformaldehyde, permeabilized with 0.5 % Triton X 100 for 15 min and stained with 2 μg/ml Phalloidin TRITC or Phalloidin FITC staining solution made in PBS for 15 min at 37 °C. Nuclei were stained with 5 μg/ml of 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) in PBS for one minute. The stained cells were mounted and images were acquired using LSM510 software on a Carl Zeiss Laser confocal Microscope at 63× magnification. The ratio of cytoplasmic/nuclear (C/N) area of approximately 100 cells was measured using Image J software to quantitate cell spreading.

Detection of MMPs by zymography and Western blotting

Melanoma cells seeded at a density of 15,000 cells in 100 μl of complete DMEM were grown in 96 well plates for 24 h at 37 °C. Cells were subjected to serum starvation for additional 24 h in absence or presence of different concentrations (0.25 μg–0.75 μg/ml) of soluble galectin-3. To see the effect of immobilized galectin-3, wells were coated overnight with 100 μl of different concentrations of galectin-3 (10–75 μg/ml) at 4 °C in serum free DMEM. The cells were grown on immobilized galectin-3 in complete medium at 37 °C for 24 h in a CO₂ incubator followed by serum starvation for 24 h. Cells seeded on uncoated wells served as control. The serum free conditioned medium was collected from each well and analyzed by gelatin zymography on 10 % SDS-PAGE containing 0.1 % gelatin as per [40]. MMP-9 levels in culture supernatant were also detected by Western blotting with anti MMP-9 antibody.

Wound healing assay

35 mm culture dishes were coated overnight with galectin-3 (50 μg/ml) in serum free DMEM at 4 °C, followed by blocking of non-specific sites with 2 % BSA for 1 h. 0.75 million melanoma cells were seeded in coated plates and incubated at 37 °C for 24 h in a CO₂ incubator. The cells were serum starved for 24 h for cell synchronization. A straight, uniform wound (approx. 400 μm in width) was
made using a micropipette tip on the monolayer and the cells were maintained in serum free DMEM. Wound closure was measured for 20 h by time lapse video imaging of at least three different positions across the length of the wound using a Carl Zeiss Inverted Microscope at 10× magnification. Uncoated culture dishes, blocked only with BSA served as control.

Cloning of shRNA for targeting β1,4 galactosyltransferases-I and -V genes (GalTs)

Downregulation of polyLacNAc in F10 cells was performed by using short hairpin RNA (shRNA) against GalT-I and -V the genes involved in polyLacNAc synthesis, as per the guidelines outlined [41]. A 21 nucleotide sequence (5'-TGGGGCGGAAGATGACGAC-3') from the open reading frame of GalT genes was chosen which is common and unique only to these two genes. The strategy for cloning shRNA into pSuperneo H1 vector is described in supplementary data.

For cloning shRNA into pTRIPz lentiviral vector, primers were designed according to pTRIPz manual (Open biosystems). Forward primer contained 

\[ 5'\text{GAACGCAGAGGTATATTGCTGTTGACAGTGAG} \]

\[ CGTGGGGCAGAAGATGACGAC \]

\[ TAGTGAAGCCACAGA}3' \]

Reverse primer contained

\[ 5'\text{GTTGAATTCCGAGGCAGTAGGCA} \]

\[ TGGGGCGGAGAAGATGACGAC \]

\[ TACATCTGTGGCTTC}3' \]

Using these primers shRNA sequence was amplified. The shRNA was cloned in pTRIPz lentiviral vector digested with EcoRI and XhoI sites. The ligated plasmid was purified and subsequently co-transfected with helper plasmids (pMD2.G and psPAX2) in HEK293FT cells for generating virus particles which were used for transduction of F10 cells. The clones were selected using puromycin (1 µg/ml) and subsequently maintained as separate stocks. For inducing shRNA expression cells were cultured in complete DMEM containing doxycycline (4 µg/ml) for 96 h.

Real time PCR

For detecting transcript levels of GalT-I and GalT-V genes specific primers were designed. RPL4 was used as housekeeping gene for relative quantification of transcript levels [42].

The real time PCR reaction was carried out in 7900HT system (ABI Prism) and for detecting amplicons Power SYBR green was used. The data represents mean of three different experiments carried out in duplicates with different batches of cDNA. The Ct values obtained were normalized to RPL4 values. Analysis was performed using 2^−ΔΔCt method [43].

Flow cytometric analysis

For flow cytometry, cells were either fixed overnight in 1.5 % glutaraldehyde or 1 % paraformaldehyde in PBS (pH 7.4) and were analyzed for surface expression of polyLacNAc using biotinylated galectin-3 (0.75 µg/ml) and biotinylated LEA (2 µg/ml) as described previously [28].

Cloning, expression and purification of mouse truncated galectin-3 (mtGal-3)

The coding DNA sequence of C-terminal carbohydrate binding domain of galectin-3 was cloned into the pET3a bacterial expression vector using forward 5'ATAGTCATCA TATCATAGTCAGTGACGATTACATGTGAAGCC ACAGA3'

Reverse primer contained EcoRI site followed by sense sequence (represented in italics bold) and loop sequence.

Reverse GalT shRNA primer

\[ 5'\text{GTGGGTACCTTTAGATCATGGCGTGGTTAGC} \]

\[ TGGGGCGGAGAAGATGACGAC \]

\[ TACATCTGTGGCTTC}3' \]

Using these primers shRNA sequence was amplified. The shRNA was cloned in pTRIPz lentiviral vector digested with EcoRI and XhoI sites. The ligated plasmid was purified and subsequently co-transfected with helper plasmids (pMD2.G and psPAX2) in HEK293FT cells for generating virus particles which were used for transduction of F10 cells. The clones were selected using puromycin (1 µg/ml) and subsequently maintained as separate stocks. For inducing shRNA expression cells were cultured in complete DMEM containing doxycycline (4 µg/ml) for 96 h.

Preparation of modified citrus pectin (MCP)

MCP was prepared from citrus pectin exactly as described in [44].

Experimental metastasis assay

Melanoma cells were routinely cultured in DMEM as described in [28], with or without glycosylation inhibitors Swainsonine (SW, 2 µg/ml) and benzyl-α-N-acetylglactosamine (BG, 2 mM). For injecting GalT clones, mice were fed with doxycycline (1 mg/ml) in 5 % sucrose solution 24 h prior to injection and continued until sacrificed.

For injecting F10 cells treated with murine truncated galectin-3 (mtGal-3, carrying only CRD), 0.1 million F10 cells were pre-incubated with 0.1 ml of 500 µg/ml of mtGal-3 for 1 h on ice. The mice that received pre-treated
cells also received injections of mtGal-3 (250 μg in 0.1 ml, via intra muscular injection) on day 1 (2 h before and after injection of cells) and once on day 2.

For injecting F10 cells treated with MCP, 0.1 million F10 cells were resuspended in medium with and without MCP (0.05 %) and mice which received cells with MCP were on drinking water containing 1.5 % MCP from 5 days prior to injection till the day of sacrifice.

**Statistical analysis**

All the data is represented as mean ± SE unless stated. For comparison of two groups in case of cell spreading, cell adhesion, experimental metastasis assay student’s t test was employed and multiple groups were compared by one way ANOVA. For wound-healing assays, 2-way ANOVA with the Bonferroni posttest was conducted. All the statistical analysis was performed using GraphPad Prism 5. P < 0.05 was considered significant.

The details of cloning of shRNA in pSupeneo H1, sequences of primers used for real time and semi quantitative RT-PCR have been described in supplementary methods. Total cell lysate, Protein estimation, SDS-PAGE and Western blotting were performed as described in [27].

**Results**

Galectin-3 is localized in all the major compartments of mouse lungs

In mice, lungs have previously been shown to express highest levels of galectin-3 and express it constitutively on the surface of its vascular endothelium. Immunohistochemistry results showed that not just endothelium, galectin-3 is localized in all the major tissue compartments of the lungs, including epithelia of bronchioles, alveoli and on the surface of vascular endothelium (Fig. 1a–f) and possibly thus may participate in different processes of organ colonization.

Galectin-3 facilitates spreading of melanoma cells

DAPI was used to stain the nuclei (blue). Spreading of F10 cells was also seen on galectin-3 coated coverslips in presence of lactose (Gal-3 Lac) and sucrose (Gal-3 Suc), and after treatment with either SW (Gal-3 SW) or BG (Gal-3 BG). Scale bar 10 μm. Each bar represents ratio of cytoplasmic to nuclear (C/N) area for around 100 cells from two different experiments. (Color figure online)
adhered on the vascular endothelium. F1 cells spread poorly with diffused organization of actin, on both uncoated and galecin-3 coated cover slips (Fig. 1g). In contrast, F10 cells showed significant spreading on galecin-3 as compared to uncoated cover slips, as characterized by organization of F-actin in lamellipodial projections and significantly increased C/N ratio (Fig. 1g, h). Like adhesion of F-actin in lamellipodial projections and galecin-3 as compared to uncoated cover slips, as characterized by organization of F-actin in lamellipodial projections and significantly increased C/N ratio (Fig. 1g, h). Like adhesion of F-actin in lamellipodial projections and galecin-3 as compared to uncoated cover slips, as characterized by organization of F-actin in lamellipodial projections and significantly increased C/N ratio (Fig. 1g, h).

Galectin-3 induces secretion of proteases and motility in melanoma cells, thereby aiding invasion

Degradation of Basement membrane/Extracellular matrix (BM/ECM) and movement are the next major event required during extravasation. Assays were performed to see if soluble and immobilized galecin-3 promotes any of these processes. Zymography of the conditioned media collected from cells grown in the absence or presence of either soluble or immobilized galecin-3, showed that galecin-3 induces secretion of matrix degrading enzyme, MMP-9 in a dose and metastatic potential dependent manner (Fig. 2a–c).

For F10 cells, the highest induction with soluble galecin-3 was seen at 0.5 μg/ml (data not shown), whereas with immobilized galecin-3, it was 2.5 μg/well of a 96 well plate (Fig. 2a). The concentrations higher than these appeared to inhibit the induction of MMP-9 secretion. Comparison of the melanoma variants clearly showed that induction is dependent on the metastatic potential (Fig. 2b, c). No gelatin clear bands (as a result of MMP-9 activity) could be visualized in F1 lanes on 24 h incubation of the gel in renaturation buffer (Fig. 2b) whereas prolonged incubation resulted in saturation in F10 lanes due to substrate limitation (Fig. 2c). Since expression of polyLacNAc on N-glycans is also dependent on the metastatic potential of melanoma cells, the galecin-3 mediated induction of MMP-9 could be via polyLacNAc.

Movement of extravasated cells towards organ parenchyma is also a key event for metastatic establishment. Wound healing assays showed that galecin-3 is indeed used as traction by these cells for their movement. This again, was dependent on the metastatic potential and the N-glycosylation status of cells (Fig. 2d–f). O-glycosylation inhibitor BG had no effect on any of these cellular properties or on metastasis [28]. This reaffirmed our earlier observation that galecin-3 ligands only on N-oligosaccharides participate in these processes.

Downregulation of GalT-I and -V enzymes in F10 cells leads to significant reduction in the expression of polyLacNAc

Although, using Swainsonine, a broad range N-glycosylation inhibitor, we confirmed that N-glycans play an important role in metastasis of F10 cells; we needed to confirm that it is via polyLacNAc on them. PolyLacNAc is synthesized by the concerted action of the enzymes that sequentially add N-acetylgalactosamine (β1,3 N-acetylgalactosaminyltransferases or β3GnTs) and galactose (β1,4 galactosyltransferases—GalTs) [45]. Among the seven members of the GalT family GalT-VII adds galactose only onto proteins with proteoglycan core [46]. Comparison of transcripts of the remaining six members by semi-quantitative PCR showed up regulation of GalT-I and -V in the higher metastatic variant (Supplementary Fig. S1A). Both β4GalT-I and β4GalT-V reportedly promote addition of polyLacNAc preferentially on N-oligosaccharides [47, 48].

Both these genes were down-regulated using shRNA targeting a sequence common to both of them, using plasmid (pSuperneo H1) as well as inducible lentiviral (pTRIPz) vectors for cloning shRNA in F10 cells. The functional effects of downregulation were confirmed by in vitro as well as in vivo assays. The two F10 cell clones, sh3 and sh6, generated in pSuperneo H1 vector which constitutively expresses shRNA showed downregulation of transcripts and polyLacNAc on the cell surface. (Supplementary Fig. S1b, c).

The inducible lentiviral vector system (pTRIPz) allowed tight temporal control of target gene knock down with minimal off-target and deleterious effects on cells. The two F10 clonal cell lines (clone I and II) expressing the inducible GalT-I and -V shRNA were established. Simultaneously, clones of F10 cells expressing the inducible non-targeting shRNA (NT) were also established. Upon doxycycline induction, clone I and II showed significant reduction in the transcript levels of GalT-I and -V as compared to clone expressing non targeting shRNA (NT) (Fig. 3a, b). In contrast to NT clone, both the clones expressing specific shRNA showed significant reduction in the surface levels of polyLacNAc as assessed by flow cytometry, using biotinylated LEA and galecin-3, after doxycycline induction (Fig. 3c, d).

Downregulation of polyLacNAc results in decreased adhesion, spreading, MMP-9 secretion and motility of F10 cells on galecin-3 together with reduced experimental metastasis

Reduced expression of polyLacNAc in the induced clone I and II was associated with significantly decreased
adhesion on galectin-3 (Fig. 3e). Downregulation of polyLacNAc also appeared to reduce their spreading on galectin-3 as compared to that of NT cells, as seen by microscopy images and C/N ratio (Fig. 3f, g). Similarly, induction of shRNA expression affected MMP-9 secretion by these clones on galectin-3 coated plates as compared

Fig. 2 Galectin-3 in both soluble (Sol Gal-3) as well as immobilized (IM Gal-3) form induces secretion of MMP-9 and IM Gal-3 promotes motility of melanoma cells. a Conditioned media of F10 cells grown on uncoated wells was compared with those grown in presence of 0.5 µg/ml sol gal-3 or on different amounts of IM gal-3 (0.1 ml/well of 10, 25, 50 and 75 µg/ml in 96 well plate). b, c Represent data comparing levels of MMPs in conditioned media of F1 and F10 cells grown on uncoated (UN, lanes 1, 4) or in presence of soluble (Sol Gal-3, 0.5 µg/ml) (lanes 2, 5) and immobilized (IM Gal-3, 0.1 ml/well of 50 µg/ml) (lanes 3, 6) galectin-3. b The data from gels incubated for 24 h, and c from gels incubated for 48 h in renaturation buffer. For quantification, densitometry analysis was performed and is represented in bar graphs below a, b, and c, respectively. d–f Represent time lapse video microscopy images at 0 and 20 h of wound closure on 2 % BSA and on IM Gal-3. Right panel depicts graphical representation of percent wound closure of d–f at 5 h interval. Mean values of triplicate for each position of the wound width of each image frame from two different experiments, was analysed using Metamorph software. * indicates P < 0.05 which was considered significant.
to NT clone (Fig. 4a, b). The motility of clones I and II on galectin-3 was also significantly reduced on induction of shRNA expression (Fig. 4c–h).

These altered cellular properties as a result of loss of polyLacNAc had a major impact on the experimental metastasis of these clones as compared to non transduced...
F10 cells or NT clones induced with doxycycline (Fig. 5a). This was also observed in the sh3 and sh6 clones generated by plasmid mediated constitutive shRNA expression. Reduced polyLacNAc levels affected galectin-3 mediated cell adhesion (Supplementary Fig. S1d), spreading and MMP-9 secretion (data not shown) resulting in significantly reduced metastatic potential of both sh3 and sh6 clones (Supplementary Fig. S1e, f).

These results collectively highlight that polyLacNAc on N-glycans on melanoma cells regulate key cellular processes that are critical for lung metastasis.

Effect of dominant negative inhibitor and the competitive sugar to galectin-3 on metastasis of F10 melanoma cells to the lungs

To confirm the role of galectin-3 as the major polyLacNAc binding lectin responsible for mediating lung specific colonization, two approaches were adopted. In the first case, all the available galectin-3 binding sites on melanoma cells were blocked using truncated galectin-3 which lacks oligomerization domain [49] and in second approach, we tried to block all the endogenous galectin-3 in mice by feeding them with MCP [44]. Both MCP and recombinant murine truncated galectin-3 (mtGal-3) were found to inhibit galectin-3 mediated spreading of F10 cells (Supplementary Fig. S2a, b). Blocking galectin-3 binding sites with excess mtGal-3 significantly reduced the metastatic potential of F10 cells (Fig. 5b). Similarly, injection of F10 cells into mice fed continuously with MCP resulted in profound decrease in their lung metastasis. MCP apparently competes with polyLacNAc on melanoma cells for binding to galectin-3 on the lungs thereby impacting metastasis (Fig. 5c).

Discussion

Interaction of specific molecules on organ endothelium and on the tumor cells is a major determinant of organ specific
metastasis [3, 50]. The strength of these interactions, ability to invade the vascular BM, entry into organ parenchyma and survival in response to organ growth environment are the other key factors that determine the organ specificity of tumor cells [3, 7]. Constitutive expression of galectin-3 on the lung endothelium apparently aids arrest of tumor cells expressing high affinity easily accessible ligands in the form of polyLacNAc on N-oligosaccharides [27]. Lungs in mice have previously been shown to express highest levels of galectin-3 [27]. Here, we demonstrate that galectin-3 is expressed not just on the endothelial cells but on all the tissue compartments of the lungs including alveolar epithelium, bronchioles and on most pulmonary tissue spaces (Fig. 1a–f).

Galectin-3 is a multifunctional nucleo-cytoplasmic protein which is involved in different cellular functions. It can interact with transcription factors in the nucleus to regulate gene expression and also can perform anti or pro-apoptotic functions depending on its cytoplasmic or extracellular localization [29]. The secreted galectin-3 often gets incorporated on the cell surface, ECM or the BM by virtue of its ability to bind to the glycoprotein ligands and oligomerise/form lattices on cell surface [51, 52].

These studies investigated if galectin-3, present in abundance in all the major compartments of the lungs, has any role in establishing metastatic foci of cells expressing high levels of polyLacNAc on N-glycans on their surface. The interactions mediated by galectin-3 are much stronger than those via selectins and are comparable to those mediated by integrins [35, 36, 53]. However, the cells adhered to the organ endothelium via galectin-3 would need to stabilize these interactions to prevent them from being flown off under hemodynamic flow conditions and to initiate processes like vascular retraction, required to extravasate. This can be achieved by initiating the spreading of adhered cells [7].

Members like galectin-8 of the galectin family in their immobilized form have earlier been shown to regulate spreading of cells [54, 55]. We for the first time demonstrate that galectin-3 in the immobilized form induces the formation of membrane protrusions in melanoma cells which can be inhibited specifically via inhibitors of N- and not O-oligosaccharides (Fig. 1e, f).

The next barrier for effective organ colonization is the exposed vascular BM. MMPs play a major role in degradation of underlying BM and facilitate tumor cell entry into
organ parenchyma [56]. Overexpression of galectin-3 in the cytoplasmic/nuclear compartments of cells has been shown to regulate expression/secretion of MMPs, especially MMP-1, MMP-2 and MMP-9 and promote invasion. Nuclear galectin-3 in gastric cancer cells was shown to interact with AP-1 transcription factor and regulate the expression of MMP-1 [57]. Further, silencing the expression of galectin-3 in human tongue carcinoma and pancreatic cell lines affected β-catenin which in turn correlated with reduced levels of MMP-2 and MMP-9 [58, 59]. Galectin-3 has collagenase like repeats adjacent to its N-terminal domain which can act as a cleavage site for MMPs and cleaved form appears to serve as a marker for cancer progression [60]. Lungs express galectin-3 in highest amounts [27] which may be present in both soluble and immobilized form in different tissue compartments including vascular BM. We show that both immobilised as well as soluble forms of galectin-3 induce secretion of MMP-9 in a dose dependent manner (Fig. 2a–c). Secretion of MMP-9 by melanoma cells correlated with their metastatic potential (Fig. 2b, c). Ours is the first study which reports that extracellular galectin-3 induces the secretion of MMP-9 in melanoma cells, most likely via the polyLacNAc on N-glycans. MMP-9 mediates degradation of ECM and not O-glycosylation status confirmed that it is indeed a mediator of N-oligosaccharides on surface glycoproteins (Fig. 2d–f). Although, other members like galectin-8 have been shown to facilitate movement of cells in a similar manner [65], here we demonstrate that even immobilized galectin-3 can facilitate haptotactic motility. Galectin-3 mediated motility would be important for cells to move into lung parenchyma.

Beta 1,6 branched expressed on N-oligosaccharides of cell surface proteins is the preferred site for further substitution of polyLacNAc. Several proteins that carry β1,6 branched N-oligosaccharides may also carry polyLacNAc. Some of the possible carrier proteins include integrin subunits (α3, α5, αv and β1), growth factor receptors like EGFR and others like CD-44 (hyaluronate receptors) and lysosome associated membrane proteins (LAMPs) [66, 67]. The cancer cells most possibly use surface receptors expressing polyLacNAc on N-oligosaccharides for motility. Galectin-3 may also promote proliferation by sustained signalling via growth factor receptors by restricting them in the lattices and preventing their internalization [68, 69].

PolyLacNAc is synthesized by the sequential addition of N-acetylgalcosamine and galactose by the enzymes β1,3 N-acetylgalcosaminyltransferases or β3GnTs and β1,4 galactosyltransferases—GalTs [45]. Among the six possible enzymes that add galactose, the expression of GalT-I and GalT-V correlated with metastatic potential of B16 melanoma cells (Supplementary Fig. 1a). Downregulation of these two genes by shRNA mediated plasmid and inducible lentiviral vectors, showed marked reduction in polyLacNAc expression (Fig. 3a, b, Supplementary Fig. 1b, c). This was accompanied with inhibition of all the galectin-3 mediated processes like adhesion, spreading, movement and induction of MMP-9 secretion (Fig. 3e–g, Supplementary Fig. 1d, 4a–h). Inhibition of experimental metastasis as a result of inhibition of all the galectin-3 mediated processes highlighted the importance of polyLacNAc and galectin-3 pair in facilitating lung colonization (Fig. 5a) (Supplementary Fig. 1e, f).

Galectin-3 is a monomeric lectin that forms oligomers on binding to its ligand via its N-terminal domain. Truncated galectin-3 devoid of the N-terminal domain has been shown to act as a dominant negative inhibitor of galectin-3. Truncated galectin-3 affected growth and lymph node metastasis of breast cancer cell line on sustained treatment [49]. Pre-incubation of B16F10 cells with truncated galectin-3 inhibited lung metastasis apparently by blocking polyLacNAc on melanoma cells making it unavailable for binding to galectin-3 on the lung vascular endothelial cells (Fig. 5b). MCP has been shown to affect several galectin-3 mediated processes including metastasis [70]. Inhibition of experimental metastasis in mice fed with MCP indicated that MCP in circulation possibly competes with polyLacNAc on melanoma cells for binding to endothelial cells (Fig. 5c). However, the contribution of galectin-3 as an immunomodulating agent also, cannot be ruled out in facilitating melanoma metastasis [32].

Our studies very clearly demonstrate the importance of galectin-3 and polyLacNAc in not just mediating adhesion to lung endothelium but also in several downstream processes critical for lung homing. It would be interesting to study the molecular pathways activated downstream
galalectin-3/polyLacNAc interactions which regulate the successive events involved in metastasis. We expect that confirmation of existence of similar mechanisms in lung metastasis of human tumors would open up several interesting avenues to explore and would also be crucial in developing effective strategies to prevent metastasis.

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Conflict of interest No potential conflicts of interest were disclosed.

References
Extracellular galectin-3 induces MMP9 expression by activating p38 MAPK pathway via lysosome-associated membrane protein-1 (LAMP1)

Manohar C. Dange · Akhil Kumar Agarwal · Rajiv D. Kalraiya

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Abstract Matrix metalloproteinases (MMPs) play a key role in matrix remodelling and thus invasion and metastasis. Extracellular galectin-3 has been shown to induce MMP9 secretion. Here, we demonstrate that galectin-3 induces MMP9 at transcript level and it is dependent on the surface levels of poly-N-acetyllactosamine (polyLacNAc). By employing signalling pathway inhibitors, MMP9 expression was shown to be induced via p38 MAP-kinase pathway. Using clones of melanoma cells expressing shRNAs to lysosome-associated membrane protein-1 (LAMP1), a major carrier of polyLacNAc, surface LAMP1 was demonstrated to serve as one of the key mediators of galectin-3-induced MMP9 expression via p38 MAPK pathway.

Keywords Galectin-3 · PolyLacNAc · MMP9 · p38 MAPK · LAMP1

Introduction

Metastasis accounts for majority of cancer-related deaths. It is a complex multistep process which involves multiple host tumour interactions [1]. To metastasize, cancer cells must dissociate from the primary, invade the surrounding normal tissue, intravasate, survive in circulation, get arrested in the target organ vasculature, extravasate and survive in the new growth environment [1, 2]. Molecules on the cell surface play a key role in these processes and tumour cells show several metastasis-associated membrane modifications [3, 4]. Expression of β1,6 branched N-oligosaccharides is one such modification [5, 6]. Their expression has been shown to correlate with disease progression and with metastatic potential in several human and murine cancer cell lines [7, 8].

Previously, we have demonstrated that substitution of these oligosaccharides with polyLacNAc promotes lung metastasis of B16 murine melanoma cells [9]. Lungs were shown to express highest levels of galectin-3 and express it constitutively on its vascular endothelium [9]. Galectin-3 on lungs was shown to not only aid circulating tumour cells to anchor on to organ endothelium, but in all the steps of extravasation. It was shown to promote spreading of cells post adhesion, degradation of vascular basement membrane (BM) and movement into organ parenchyma [10]. PolyLacNAc only on N- and not O-oligosaccharides was shown to participate in these processes and even role of other ligands such as T/Tn antigens in melanoma metastasis was ruled out [11]. β1 integrin and the lysosome-associated membrane protein 1 (LAMP1) have been identified to be the major carriers of polyLacNAc on melanoma cells [9].

Expression of LAMP1 on the melanoma cell surface has been shown to correlate with the metastatic potential, and downregulation of its expression inhibits its surface expression and impacts cellular properties like spreading, movement on immobilized galectin-3 and metastatic potential [9, 12]. We for the first time demonstrated that galectin-3 also induces secretion of MMP9 in these melanoma cells in a metastatic potential-dependent manner. Galectin-3 in both soluble and immobilized/coated form was shown to induce secretion of MMP9 in high metastatic...
melanoma (B16F10) cells in a concentration-dependent manner. Inhibition of polyLacNAc on these cells inhibited secretion of MMP9 [10].

Present investigations reveal that galectin-3-induced secretion of MMP9 is regulated at the transcriptional level by the extent of polyLacNAc on the cell surface. The signalling pathway and the polyLacNAc carrying protein through which galectin-3 transmits these signals have been identified in these studies.

Materials and methods

Reagents

_Escherichia Coli_ BL 21 with pET3C plasmid containing a full-length recombinant human galectin-3 was a kind gift from Prof. Hakon Leffler, Lund University, Sweden. TRIzol was from Invitrogen, USA. Power SYBR Green PCR Master Mix and high capacity cDNA reverse transcriptase kit were from Applied Biosystems, Life technologies, USA. Primers for real-time PCR and shRNA cloning, polybrene, anti-mouse antibody for MMP9 raised in rat were purchased from Sigma Chemical Company, USA. Anti LAMP1 antibody (clone 1D4B) raised in rat was from BD Biosciences, USA. Anti-goat HRPO was from Santa Cruz Biotechnology, USA. Inhibitors for PI3K (Wortmannin), ERK (PD169316), p38 MAPK (SB203580) and JNK pathways, protease inhibitor cocktail and phosphatase inhibitor cocktail were from Calbiochem, USA. pTRIPz vector and packaging vectors (pMD2.G and psPAX2) were from Open Biosystems, USA. Dulbecco’s Modified Eagle’s Medium (DMEM) and Foetal Bovine Serum (FBS) were purchased from Gibco, Invitrogen, USA. All other chemicals were purchased locally and were of analytical grade.

Cell lines

B16F10 (F10) murine melanoma cell line obtained from the National Centre for Cell Science, Pune, India was cultured, stored and maintained as described in [9]. Poly-LacNAc downregulated clones and LAMP1 downregulated clones were validated as described in [10] and in [12]. For induction of shRNAs, cells were grown in complete medium containing doxycycline (4 µg/ml) for 96 h. The inducible lentiviral vector system (pTRIPz) allowed tight temporal control of target gene knock down with minimal off-target and deleterious effects on cells.

Purification of recombinant human galectin-3

Galectin-3 was purified as described in [11].

Detection of MMPs by gelatin zymography and Western blotting

Gelatin zymography was performed as described in [13]. 60-mm plates were coated overnight at 4 °C with 2 ml either of 10, 25 or 50 µg/ml galectin-3 or of 10 µg/ml fibronectin. For blocking signalling pathways, equal number of cells were seeded on galectin-3-coated plates and grown till 50 % confluency for 24 h in the presence of serum-containing DMEM medium. Cells were then gently washed thrice with plain DMEM and were incubated in serum-free DMEM containing required concentrations of signalling inhibitors. DMSO was used as vehicle control. Culture supernatant collected after 24 h (conditioned medium) was then subjected to either zymography or Anti-MMP9 immunoblotting as described in [10]. Also, to assess if inhibitor treatment had any effect on cell viability, cells remaining in the plates were lysed in 1X reduced sample buffer and equal volumes of lysates from each sample were resolved by SDS-PAGE, proteins were transferred onto PVDF membrane and probed with β actin antibody or stained with coomassie brilliant blue. All the experiments were repeated in triplicates, and quantitation by densitometric analysis (for zymography experiments) was performed as described in [14].

Real-time PCR

The cells remaining in culture plates after collection of conditioned medium were subjected for RNA preparation by TRIzol solution. 1 µg of RNA was then used for preparation of cDNA using high capacity cDNA reverse transcriptase kit. The real-time PCR reaction was performed as described in [10]. RPL4 was used as housekeeping gene for relative quantification of MMP9 transcript levels. The data represent mean of three independent experiments carried out in duplicates with different batches of cDNA. Analysis was performed using 2^(-ΔΔCt) method [15].

The sequence of primers (left to right in 5’ to 3’ direction) used for amplification is as follows:

- MMP9 forward primer-TCATTCCGCTGGATAAGG
- MMP9 reverse primer-AGGCTTTGTCTTGGTACTGG
- RPL4 forward primer- GACAGCCCTATGCCGTCA
- RPL4 reverse primer- GCCACAGCTCTGCCAGTACC

Preparation of cell lysates for detection of phosphorylated signalling proteins by immunoblotting

Cells were grown on galectin-3-coated plates as described in previous method. After growing under serum-free conditions for 24 h, cells were harvested in lysis buffer
containing protease and phosphatase inhibitor cocktail. Cells were then sonicated and centrifuged at 16,000 rpm for 30 min at 4 °C. The protein concentration was estimated as described in [9]. 100 μg of protein was mixed with Laemmli buffer, boiled for 5 min and loaded on SDS-PAGE under reducing conditions. The proteins were transferred on PVDF membrane and probed with phosphospecific p38 MAPK antibody and blots probed with p38 MAPK antibody served as loading control.

Statistical analysis

All the data are represented as mean ± SE unless stated. For comparison of two groups, student’s t test was employed. All the statistical analysis was performed using GraphPad Prism 5. P < 0.05 was considered significant.

Results and Discussion

Matrix metalloproteinases (MMPs) are the key molecules involved in cancer cell invasion and tissue remodelling [16]. MMPs are tightly regulated at multiple levels as it may impact tissue integrity [17]. Multiple mechanisms have evolved to regulate the action of MMPs [18]. It can be at the level of its transcription, expression, secretion, activation of pro form to active form, localization at the invading front and interaction with its tissue inhibitors [19]. The primary regulation is at the mRNA level and under normal physiological conditions; only basal levels of MMP transcripts are produced. In response to external stimuli such as growth factors, cytokines and extracellular matrix (ECM) components, the expression of MMPs is upregulated [20].

Galectin-3 is a nucleo-cytoplasmic protein that also gets secreted in a non-classical manner. The secreted galectin-3 can get immobilized on the cell surface or can become a part of the ECM and BM [21, 22]. Extracellular galectin-3 has been shown to regulate processes associated with invasion and metastasis [23]. Recently, we have shown that both soluble and immobilized form of extracellular galectin-3 induce MMP9 secretion, which appear to depend on the metastatic potential of the cells and the levels of polyLacNAc expression on their surface [10]. Does galectin-3 induce secretion of MMP9 by inducing the transcription of MMP9 and what are the signalling pathways induced.

Extracellular galectin-3 induces MMP9 expression at mRNA level via p38 MAPK pathway

Using zymography it was shown that extracellular galectin-3 in immobilized form induces secretion of MMP9 in culture supernatants of F10 cells in a dose-dependent manner. The maximum induction occurred in cells grown on plates coated with 50 μg/ml of galectin-3 (Fig. 1a). Fibronectin, a well-studied ECM protein and a known inducer of MMP9 secretion [24, 25], served as a positive control (Fig. 1a). The levels of MMP-9 mRNA in cells grown on uncoated plastic plates were compared to those grown on fibronectin or galectin-3-coated plates by real-time PCR to determine if induction occurs at the transcript level. Cells grown on galectin-3-coated plates expressed significantly increased levels of MMP9 transcripts as compared to cells grown on uncoated plates (Fig. 1b). These results suggest that galectin-3 in extracellular form can indeed induce the transcription of MMP9. Our results are also corroborated by the recent findings which highlight the role of extracellular galectin-3 in induction of MMP9 at transcript level in migrating epithelial cells [26].

ECM proteins regulate the cellular signalling pathways involved in matrix remodelling by interacting with their receptors on the cell surface like integrins to initiate outside in signalling and vice versa [19]. In response to external stimuli, cellular signalling pathways are activated which then modulate the transcription of MMPs. ECM proteins such as fibronectin and osteopontin are known to activate MMP9 expression through ERK, PI3K or NF-κB pathways [25, 27]. To understand which downstream pathways are activated by extracellular galectin-3, inhibitors of PI3K and MAPK (ERK, p38 MAPK and JNK) pathways were used.

Comparison of transcript levels of MMP9 in cells grown on galectin-3-coated plates in the absence or presence of different signalling inhibitors showed statistically significant reduction in transcript levels of MMP9 only in the presence of ERK and p38 MAPK inhibitor (Fig. 1c); however, the maximum inhibition was seen in the presence of the latter. Transcript levels of MMP9 in cells grown on galectin-3-coated plates treated with vehicle alone served as control.

These results were further corroborated upon evaluation of MMP9 levels in the conditioned medium collected from cells grown on galectin-3-coated plates in the absence or presence of different signalling inhibitors by zymography and immunoblotting. Both, confirmed that in comparison to cells grown in the presence of ERK, PI3K and JNK inhibitors, cells treated with p38 MAPK inhibitor (SB) showed maximum reduction in MMP9 levels (Fig. 1d–f). Western-blotted lysates of untreated and inhibitor-treated cells were probed with β actin antibody or stained with coomassie brilliant blue which served as control for equal number of cells (Supplementary Fig. 1a, b).
Downregulation of polyLacNAc, the high affinity galectin-3 ligand, inhibits induction of MMP9 expression in melanoma cells

Repeating units of galactose and N-acetylglucosamine, Poly-N-acetyllactosamine (polyLacNAc), is synthesized by the concerted action of β1,4 galactosyltransferase and β1,3 N-acetylglucosaminetransferase [28]. PolyLacNAc is the preferred ligand for galectin-3 and the strength of its binding is dependent on the number of LacNAc repeats [29]. The expression of polyLacNAc on N-glycans on melanoma cells correlates with their metastatic potential. Inhibition of expression of N-glycans by Swainsonine, or the β1,6 branch by inhibiting the expression of the enzyme GnT-V inhibits experimental metastasis [11, 13]. Beta 1,6 branched N-oligosaccharides are often substituted with polyLacNAc. Downregulating the enzymes involved in polyLacNAc synthesis not only affected binding of galectin-3 to melanoma cells but also galectin-3-mediated processes, including cellular spreading, motility and matrix degradation. Inhibition of these cellular processes ultimately affected metastatic potential of melanoma cells [10]. In light of these findings, it was important to understand if galectin-3-induced transcription of MMP9 is indeed through polyLacNAc. We have used inducible lentiviral shRNA clones of F10 cells in which genes for the enzymes involved in polyLacNAc synthesis (GalT-I and -V) were downregulated [10]. Reduced surface polyLacNAc in these clones not only...
affected MMP9 induction at protein level [10] but also at the mRNA level (Fig. 2a). Downregulation of GalT-I and V did not affect the fibronectin-induced MMP9 expression as seen at transcript (Supplementary Fig. 2a) as well as at protein levels (Supplementary Fig. 2b, c) which clearly highlights the specific role of polyLacNAc in galectin-3-induced MMP9 secretion.

If inhibition of p38 MAPK pathway affects MMP9 induction (Fig. 1c–f), then it would be interesting to investigate if the activation of the same pathway is hampered in polyLacNAc downregulated clones. Both the clones upon doxycycline induction showed significantly reduced levels of phospho-p38 MAPK, suggesting that polyLacNAc also signals galectin-3-mediated processes via this pathway (Fig. 2b, c).

LAMP1 and β1 integrin are among the major proteins identified to carry polyLacNAc substituted β1,6 branched N-oligosaccharides on these melanoma cells [9]. LAMP1 is a highly glycosylated protein that lines the lysosomes [30]. More than 60% of its weight is contributed by carbohydrates and each molecule carries about 17–20 N-glycans that are highly substituted [30, 31]. In metastatic cells, LAMP1 is known to get translocated to the cell surface [9, 32]. The extent of its surface expression and the levels of polyLacNAc on N-glycans have been shown to correlate with the metastatic potential of melanoma cells [9]. In addition, glycosylation in these cells has been shown to modulate the surface expression of LAMP1 [33]. LAMP1 is reportedly a known ligand for galectin-3 [34, 35]. Recently, downregulation of LAMP1 expression has been shown to significantly affect its surface expression, as well as the spreading and motility on galectin-3 and experimental metastasis of F10 cells [12]. It is thus possible that LAMP1 is one of the molecules involved in galectin-3-mediated signalling that induces MMP9 transcription and secretion.

LAMP1 participates in galectin-3-mediated induction of MMP9 expression via p38 MAPK pathway

To investigate the role of LAMP1 in MMP9 expression, melanoma cells expressing shRNAs for LAMP1 were used [12]. Real-time PCR results show that downregulation of LAMP1 in F10 cells (clones Sh1 and Sh2) considerably affects the galectin-3-induced transcription of MMP9 (Fig. 3a). This is further reflected by significantly reduced secretion of MMP9 in LAMP1 downregulated clones grown on galectin-3, as analysed by zymography (Fig. 3b, c) and immunoblotting (data not shown) suggesting that LAMP1 can be one of the upstream regulators in MMP9 induction. This was also corroborated by decreased levels of activated (phospho) p38 MAPK in the lysates of LAMP1 downregulated clones (Fig. 3d, e). However, these cells did not show any significant change in the levels of the MMP9 or its transcript when grown on fibronectin (Supplementary Fig. 2d–f), suggesting that the signalling event on galectin-3 is specifically via carbohydrate/lectin interaction (Fig. 4).

LAMP1 has a very short cytoplasmic tail (consisting of only eleven amino acids) [30] and has few known binding partners. Ezrin, a member of ERM family of proteins, is one such protein that has been shown to interact with LAMP1 at the cytoplasmic end [36]. Ezrin can function as a linker between membrane proteins and cytoskeletal proteins to modulate cellular adhesion and motility [36–38]. The loss of spreading and motility on galectin-3 in LAMP1 downregulated clones [12] is possibly via some such mechanism. It is possible that induction of MMP9 by surface LAMP1 is also mediated by similar protein(s) that interact with LAMP1 and activate the downstream
Fig. 3 Downregulation of LAMP1 affects galectin-3-induced MMP9 expression via p38 MAPK pathway. a Bar graph represents analysis of levels of MMP9 transcripts by real-time PCR for NT and LAMP1 downregulated clones Sh1 and Sh2, grown on galectin-3-coated plates in the absence (−D) and presence of (+D) doxycycline. b Levels of MMP9 in culture supernatants of NT, Sh1 and Sh2 cells grown in the presence (+D) and absence (−D) of doxycycline on galectin-3 as detected by zymography. c Densitometric analysis of bands in (b) by Image J software. d Western blotting for detection of phospho-specific forms of p38 in NT, Sh1 and Sh2 clones (−D and +D) grown on galectin-3-coated plates. Blots probed with p38 MAPK antibody served as loading control. e Densitometric analysis of bands in (d) by Image J software.

Fig. 4 Schematic representation summarizing the overall events involved in galectin-3 induced MMP9 expression in melanoma cells signalling pathways leading to induction of MMP9 transcription and secretion.

Alternatively, as a glycosylated cell surface protein, high levels of polyLacNAc on LAMP1 may initiate/contribute to formation of galectin-3-mediated lattices/membrane microdomains. These microdomains may also include other receptors like integrins (α5, α3, αV and β1), cadherins, growth factor receptors such as epidermal growth factor receptor (EGFR), TGF β, etc., [39–43]. The signalling may thus be indirectly mediated through such components of the lattice. LAMP1 may thus be possibly controlling the signalling by regulating the formation of the lattice. It would be interesting to explore such mechanisms which can play an important role in regulating key cellular processes such as matrix degradation.

Our study describes the novel function of extracellular galectin-3 and also explores the downstream signalling mechanisms of galectin-3/polyLacNAc pair which are not yet completely elucidated. In a broader sense, such studies in tumour cell biology can throw light on some of the most intricate mechanisms by which invasion and metastasis is regulated at the molecular level.

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