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Radiation-induced Galectin-1 by endothelial cells: A promising molecular target for preferential drug delivery to the tumor vasculature

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Abstract

The present study reports on a new strategy for selective, radiation therapy-amplified drug delivery using an antiangiogenic 33-a.a., tumor vasculature targeting ligand, anginex to improve the therapeutic ratio for strategies developed against solid tumors. Our findings indicate that Galectin-1 is (i) one of the major receptors for anginex (ii) over expressed by tumor neo-vasculature; and (iii) further specifically upregulated in endothelial cells in response to radiation exposure as low as 0.5 Gy. An investigation of [18]-F-labeled anginex biodistribution in SCK tumors indicates that anginex is an effective targeting molecule for image and radiation-guided therapy of solid tumors. An anginex-conjugated liposome capable of being loaded with drug was shown to selectively target endothelial cells post-radiation. The presence of endothelial cells in a three dimensional co-culture system with tumor cells developed to study tumor/endothelial cell interactions *in vitro* led to higher levels of galectin-1 and showed a further increase in expression upon radiation exposure when compared to tumor cell spheroids alone. Similar increase in galectin-1 was observed in tumor tissue originating from the tumor-endothelial cell spheroids *in vivo* and radiation exposure further induced galectin-1 in these tumors. The overall results suggest feasibility of using a clinical or subclinical radiation dose to increase expression of the Galectin-1 receptor on the tumor microvasculature to promote delivery of therapeutics via the anginex peptide. This approach may reduce systemic toxicity, overcome drug resistance and improve the therapeutic efficacy of conventional chemo/radiation strategies.

Keywords

Galectin-1; endothelial cells; tumor vasculature; anginex; tumor-endothelial cell spheroids

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Introduction

One of the primary goals of a successful cancer treatment regimen is to deliver an effective combination of radiation and/or drugs to tumors while minimizing damage to normal tissues. Tumor growth is essentially considered to be directly dependent on its blood supply. Several unique proteins functionally important to tumor angiogenesis are expressed on tumor endothelial cells and can serve as potential targets for drug delivery to solid tumors. Radiation has also been found to achieve site specific expression or upregulation of receptor(s) within tumors which are now being considered as ligands for targeted drug delivery (1, 2). Since approximately 50–60% of patients receiving radiation receive concomitant chemotherapy at present with this number continuously increasing, targeting drug delivery to the tumor vasculature by radiation-induced receptor expression on endothelial cells is a very promising approach. The 14.5 kDa protein, Galectin-1 shown to be involved in tumor angiogenesis has been found to be upregulated in the tumor microenvironment(3) of colon(4), prostate (5), bladder (6), and breast (7) cancers. Our recent findings establish that the already elevated galectin-1 expression in the tumor stroma further increases, after radiation exposure, particularly on the endothelial cell surface.

Targeting the tumor vasculature is a strategy that can therefore allow targeted delivery to a wide range of tumor types (8, 9). The past two decades has given rise to an emerging literature establishing galectin-1 as an important protein in cancer biology that is enriched in the tumor associated neovascular endothelium (10–13) and has identified the 33 amino acid, antiangiogenic anginex peptide (14, 15) to specifically bind and inhibit the function of galectin-1 receptor (11–13, 16). The dual targeting effect of anginex and $\alpha(v)\beta(3)$ integrin RGD, another angiogenesis specific ligand has recently been reported for molecular imaging of angiogenesis (17, 18) and has indicated the potential for targeted drug delivery. We are the first, to our knowledge, to elucidate in this study the effect of radiation exposure on induction of galectin-1 expression in the endothelial cells and the tumor vasculature *in vivo*. We have observed an induction in galectin-1 expression at radiation dose as low as 0.5 Gy. Such low subclinical doses of ionizing radiation may be effectively used to ‘prime’ the target, galectin-1 in the tumor associated endothelium for drug delivery via anginex or galectin-1 specific antibody. Our present and earlier studies with [18]-F-labeled anginex biodistribution and anginex-conjugated liposome uptake suggested that anginex could be an effective targeting molecule for image and radiation-guided therapy of solid tumors (19, 20). We therefore hypothesized that delivery of nanosized chemotherapies via the anginex peptide targeted to radiation-induced, tumor-vasculature associated Galectin-1 would allow for preferential targeting to the tumor site.

We have also studied the expression of galectin-1 upon radiation exposure in a three dimension co-culture system of tumor and endothelial cells grown in hanging drops of medium that more closely simulates the tumor/tumor microenvironment *in vivo*. Presence of endothelial cells in the co-culture enhances the expression of radiation-induced galectin-1 and protects tumor cells from radiation-induced cell death. (21). Similar pattern of galectin-1 expression is observed when these tumor–endothelial co-cultures are implanted *in vivo*. Future work will incorporate this murine breast cancer model in developing our strategy for radiation guided drug delivery. Results from the current study lay the foundation for the development of a chemoradiation strategy where we can exploit clinically relevant radiation doses for enhanced drug delivery with reduced side-effects and co-morbidities and an improved therapeutic ratio.

Materials and Methods

Antibodies and reagents

The Galectin-1 (sc-19277) and actin (sc-1616) antibodies were from Santa Cruz Biotechnology and the CD34 (Cat. No. 553731) antibody was from BD Pharmingen. Anginex was obtained from the biochemical facility of University of Minnesota as a lyophilized powder and reconstituted in water for *in vitro* experiments or methanol for PET imaging. All other solvents or reagents were purchased from Fischer Scientific and Sigma-Aldrich unless otherwise noted. The ProteoExtract Native Membrane Protein Extraction Kit (Cat. No. 444810) was purchased from Calbiochem.

Cell lines and culture

The EA.hy926 human and 2H11 murine endothelial cell lines and MDA-MB-231 human breast adenocarcinoma cell line were obtained from American Type Culture Collection. Human umbilical vein endothelial cells (HUVEC) were bought from Lifeline Cell Technology. Isolation and culture of Microvascular endothelial cells (MVEC) was as described (22). The endothelial progenitor cells (EPC) were isolated and cultured as described (23). GFP-4T1 murine epithelial carcinoma and the SCK murine mammary carcinoma cell lines were kind gifts from, Dr. Alexander Asea (Texas A & M Health Science Center, Temple, TX) and Dr. C Song (Masonic Cancer Institute, Minneapolis, MN) respectively. The cell lines were maintained in monolayer culture at 37 °C and 5% CO₂. The MDA-MB-231, GFP-4T1 and 2H11 cell lines were cultured in Dulbecco's minimal essential medium (DMEM, Cellgro), supplemented with 10% fetal bovine serum (Atlas), 1% penicillin/streptomycin (Hyclone). The EA.hy926 cells were maintained in DMEM/F12 with 10% Bovine calf serum, 2% Hypoxanthine Aminopterin Thymidine (HAT) medium (Cellgro). The SCK tumor cell line was grown in RPMI 1640 (Hyclone) and supplemented with 10% bovine calf serum (Atlas Biologicals), 1% penicillin/streptomycin (Hyclone).

Membrane and whole cell lysates

Whole-cell extracts were prepared by suspending cells in 0.25 ml of lysis buffer (25 mM HEPES, pH 7.5, 0.5% sodium deoxycholate, 5 mM EDTA, 5 mM dithiothreitol, 20 mM - glycerophosphate, 1 mM Na₃VO₄, 50 mM NaF, 1% Triton X-100, 20 µg/ml aprotinin, 50 µg/ml leupeptin, 10 µM pepstatin, 1 µM okadaic acid, and 1 mM phenylmethylsulfonyl fluoride). The lysates were incubated at 4°C with gentle agitation on a rocker plate (Mini Mixer, Benchmark Research Products, NY) for 1 hr and cell debris was removed by centrifugation (15 min at 12,000 × g). The protein concentration in the supernatant was determined using the BCA protein assay kit (Pierce).

Extraction of Membrane Fraction

The membrane protein fraction was extracted using the Native Membrane Protein Extraction kit (Calbiochem) according to the manufacturer's suggested protocol. The supernatant-containing membrane protein was collected and stored at -80°C for future analysis. Protein concentrations of the membrane fraction obtained were determined using the BCA protein assay kit (Pierce).

Western blot analysis

Immunoblotting was performed with an antibody against galectin-1 and actin (Santa Cruz Biotechnology, CA) and the CD34 (BD Pharmingen).

Spheroid culture in “Hanging drop”

GFP-4T1 tumor cells and 2H11 endothelial cells were used to generate multicellular spheroids by growing them as ‘Hanging drops’ of medium (in DMEM with 10% FBS and antibiotic/anti-mycotic mix) (21).

Animals

Female, athymic, nude mice (CrI: NU(NCr)-Foxn1^{nu}) 8–9 weeks old and 20–22 g were purchased from Charles River Laboratories (Wilmington, MA) and housed in the UAMS animal care facility. All experiments were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas for Medical Sciences. The mice were given clean water *ad libitum* and 2016 Teklad Global 16% Protein Rodent Diet (Harlan Feeds, Woodland, CA).

SCK tumor implants in Rear limb of athymic nude mice

SCK mouse mammary carcinoma cell were cultured in RPMI 1640 medium (Hyclone, Logan, UT) supplemented with 10% bovine calf serum (Atlas Biologicals, Fort Collins, CO). Cells were harvested in log phase with 0.125% trypsin (Mediatech Inc., Herndon, VA), counted with a Z2 Coulter Counter (Beckman Coulter, Brea, CA), spun, and resuspended in serum-free medium at a concentration of 3×10^5 cells/50 μ l. The tumor cells were injected in the right hind limb above the ankle. Tumor growth was recorded by caliper measurement. The mean of two perpendicular diameters was obtained. Mice were treated and imaged when the tumors grew to 8–10 mm size.

Experimental radiation

Radiation was carried out in CP 160 X-ray system (Faxitron X-ray Corporation Tucson, AZ, USA). The instrument may be operated at different kVps, shelf height (SSD), filtration thickness and electrically operated turntable to ensure uniform dosing. For all experiments shelf-6 (SSD= 43.2 cm covering ~39 cm diameter field, 0.8 mm Be/ 0.5 mm Cu filtration,) was used with 150 kVp, 6mA beam. Dosimetry was carried out using a pin-point ion chamber (PTW N301013, ADCL calibrated for 225 kV) following the AAPM TG-61 protocol (24). The radiation dose rate in our set up was (1.018 ± 0.10) Gy/min at 150 kV and 6.6 mA. Mice were anesthetized with isoflourane and flank irradiation was carried out with a custom cut lead shielding jig covering the animal, except for the tumor bearing extremity.

Synthesis of [¹⁸F]anginex with [¹⁸F] fluorobenzaldehyde

Synthesis of [¹⁸F]fluorobenzaldehyde was done as previously reported (19). It was then used to label anginex via reductive amination as described (19).

MicroPET —SCK tumors

To detect the [¹⁸F]anginex uptake by the SCK tumor 2 h static microPET imaging was performed in five tumor graft-bearing mice after injection with 1.1 MBq (30 μ Ci) of [¹⁸F]anginex tracer in a solution of 10 mg/ml of unlabeled anginex. Static scans of the tracer using UAMS microPET Focus 220 (Concorde Microsystems, Knoxville, TN) were captured for 20 min through the tumor region before and after the radiation exposure. PET data were iteratively reconstructed with the ordered-subsets expectation maximization (OS-EM) algorithm. The tracer uptake was reported as mean standardized uptake values (SUV), which was calculated as the radioactivity of the region of interest (ROI) divided by the injected dose per animal body weight. Activities within the tumor were normalized to the total body activity.

Preparation of Anginex conjugated fluorescent liposomes

Liposomes with a diameter of approximately 150 nm that were prepared had a basic formulation consisting of DSPC (1,2-distearoyl-*sn*-glycero-3-phosphocholine), Cholesterol, and DSPE-PEG (Amine 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-Dapa), or DSPE-PEG Maleimide (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000]), with molar ratios 1.1:1:0.075. The liposomes were rendered fluorescent by addition of ~0.1 mol % fluorochrome (TexasRed-1, 2-dihexadeconoyl-*sn*-glycero-phosphoethanolamine). The liposomes were prepared by extrusion through nuclear filters with the appropriate pore size. A low immunogenic response was assured by the PEG-functionalization of these lipids. The liposomes size distribution was analyzed by using a Dynamic Light Scattering particle size analyser (Brookhaven Instruments Corporation, Holtsville, NY). The liposome functionalization with Anginex peptides was made using the maleimide moieties attached to the end of the DSPE-PEG-Maleimide lipids, and the additional Cysteine at the Anginex N-terminal. The maleimide group reacts specifically with sulphhydryl groups of the peptide and forms a stable thioether bond. The unreacted peptide was removed by overnight dialysis. The presence of Anginex on the liposomal surface was established by Zeta Potential measurements (Zeta-Pals facility, University of Arkansas, Fayetteville, AR).

Flow cytometry

Acquisition and analysis of data was performed using an EPICS1XLTM flow cytometer (Beckman Coulter, Fullerton, CA). For each cell line, viable cell size scatter dot plot was gated for control non targeted liposome sample and bars were set in FL3 channel for background area, shifted area and whole area, protocol was saved and applied to all samples in the experiment. The x-median 50 values of the whole area was used for comparisons.

Diaminobenzidine tetrahydrochloride (DAB) immunohistochemistry

Tumors were fixed in 10% Neutral buffered formalin (NBF) and embedded in paraffin. 5 μ m sections were immunostained with Hypoxyprobe-1 kit (HPI, Burlington, MA) according to manufacturer recommendations. Tissue imaging was performed using an Aperio Scanscope (Aperio, Vista, CA) at 20 $\times$ magnifications and analyzed using Imagescope software (Aperio, Vista, CA). Necrotic areas, tissue folds and borders were excluded from analyses and remaining viable tissue was analyzed for DAB using an algorithm preconfigured to quantify brown color in three intensity ranges (weak, medium and strong).

Statistical Analysis

The results are expressed as the means $\pm$ S.D, and the significance of differences was evaluated by Student's *t*-test using SPSS (version 15.0) software (SPSS Inc., Chicago, IL, USA). Values were considered statistically significant when $P < 0.05$.

Results

Elevated expression of Galectin-1 in tumors from different tissue origin and MicroPET imaging of anginex uptake by SCK tumors which is further augmented upon radiation exposure

To investigate the expression pattern of Galectin-1 in human tumor and normal tissues and establish the validity of our targeting strategy, human lung, bladder and breast tumor specimens and the corresponding normal tissues were examined by western blot analysis. A 4-, 4.75- and 2.2-fold increase in galectin-1 expression was observed in the lung, bladder and breast tumor tissues respectively when compared to their corresponding normal tissues Figure 1A.

We have developed a method to label anginex with [18]-F and established its use for imaging of tumors in myeloma models (19). Figure 1B demonstrates anginex uptake before and after a typical clinical radiation dose of 2Gy in murine mammary SCK tumors *in vivo* using the microPET imaging strategy. Radiation exposure increased uptake by an average of 141 $\pm$ 49% a substantial increase in terms of clinical PET [significance is typically regarded as a 30% change in standardized uptake value in solid tumors (25)]. Figure 1C shows a time course of anginex uptake by the SCK tumor and normal muscle tissue in A/J mice. The A/J mice were injected with 0.86 mCi of total activity with a significant amount taken up by the tumor tissue over the first 45 min post injection.

We have also generated new evidence in the laboratory that human tumor xenografts in SCID mice express Galectin-1, and that treatment with anginex and/or radiation modifies its expression in the tumor [Figure 2A] (20). Digital quantification of the images in Figure 2B showed a ~50% increase in the positive staining upon radiation exposure (**upper panel**) and a ~10-fold increase in strong positive staining for Galectin-1. Addition of anginex treatment to radiation markedly reduced the amount of staining (**lower panel**) indicating the inability of the antibody to bind to Galectin-1 because of its binding to anginex and/or the destruction of cells expressing gal-1 after anginex and radiation treatment. This competitive binding of anginex and Galectin-1 antibody or loss of Galectin-1 expression caused reduced immunostaining of the tumor with Galectin-1 antibody.

Targeting of anginex-conjugated liposomes to tumor and endothelial cells upon radiation exposure via anginex-galectin-1 binding

We have found increased expression of Galectin-1 in the membrane fractions of different human endothelial cell types [Figure 3A and 3B with loading control in Supplemental Figure 1] and 2H11 murine endothelial cell line [Figure 3C] upon exposure to radiation doses as low as 0.5 Gy. This increase is however, not observed in the breast cancer cell line, MDA-MB-231 [Figure 3A, **last panel**]. A liposomal formulation including fluorescently tagged lipids and a covalent linkage using maleimide of bioactive anginex (26) on the surface was developed as illustrated in Figure 4A. Studies with 'anginex-tagged' liposomes subsequently indicated significant binding and uptake of the carrier by endothelial and tumor cells in culture. There was a gradual increase in uptake of anginex-conjugated fluorescent liposomes with increasing dose and duration of incubation with 2H11 endothelial cells [Figure 4B]. Figure 4C shows the difference in binding of anginex tagged liposomes (TL) to 2H11 endothelial cells compared to liposomes only (NTL). This binding of the anginex-tagged liposomes increased specifically on endothelial cells after exposure to 2 and 4 Gy radiation [Figure 5A and 5B] and not in the SCK tumor cells upon radiation exposure, supporting observations made with our *in vitro* results. Non-tagged liposomes exhibit little to no binding to either cell type before or after radiation. Furthermore, the addition of either free anginex or anti-Galectin-1 antibody to the 2H11 endothelial cells along with the targeted liposomes blocked uptake by ~50% in general and specifically blocked the radiation-induced uptake in the 2H11 endothelial cells, suggesting a Galectin-1 mediated pathway for anginex-conjugated liposome binding [Figure 5C and 5D]. This correlated with our previous results that indicated expression of Galectin-1 increases in response to radiation in the membranous fraction of the 2H11 murine endothelial cell type [Figure 3C].

Elevated Galectin-1 in murine tumor endothelial cell spheroids that is further enhanced upon radiation

In the three dimensional multicell co-culture system that we have developed to capture the interaction between tumor and endothelial cells, we investigated the response of Green Fluorescent Protein (GFP) expressing 4T1 mouse mammary tumor cells and the 2H11

murine endothelial cells to radiation exposure. Figure 6A shows a GFP expressing 4T1-2H11 tumor-endothelial cell spheroid 10 days post culture in a hanging drop of medium (i and ii). The Galectin-1 expression was found to be elevated in 4T1-2H11 tumor-endothelial spheroids as compared to 4T1 tumor cell only spheroids which was further enhanced in the tumor-endothelial 4T1-2H11 cell spheroids upon exposure to 4Gy of radiation (Figure 6B). This galectin-1 expression was also found to be elevated in lysates of tumors originating from the tumor-endothelial cell spheroids growing in window chamber implants of nude mice (Figure 6C). Induction of galectin-1 expression upon radiation exposure was also observed in tumors generated from tumor-endothelial cell spheroids in the rear limb of nude mice (Figure 6D).

Discussion

Galectin-1 expression has been found to be elevated in inflamed and neoplastic tissues. It is expressed on the surface of activated endothelium, stromal fibroblasts, tumor cells, and antigen presenting cells of the neoplastic tissue (5, 27–30). Studies reported in literature indicate that Galectin-1 is over expressed in the tumor and surrounding tissue, ‘the tumor stroma’ and its increase is related to tumor progression, immune-escape and metastasis (3). However, because it is regulated by several factors, its expression levels in different cell types constituting the tumor/tumor microenvironment tend to vary. Hypoxia inducible factors like HIF-1 α (31, 32), soluble factors secreted by tumor cells (28) and radiation inducible factors which might also be endothelial cell specific like Egr-1 (33, 34) may also play a role in enhancing the expression of galectin-1 on the endothelial cell surface. A schematic depicting the possible regulation of galectin-1 in tumor and endothelial cells by radiation is shown in Supplemental Figure 2. It is possible that the effects of radiotherapy on the tumor microenvironment maximally stimulate the signaling pathways involved in galectin-1 expression, secretion, and possibly function on the inside, outside or even distant from the tumor cell. For instance, the expression of HIF-1 α has been clearly established to be stimulated in endothelial cells by the stress of ionizing radiation exposure (35) and is a driver of galectin-1 expression (36). Furthermore, the idea that galectin-1 might be used by tumor cells to promote progression and growth has been elegantly established by Griffioen and his group(10). Therefore, our results suggest that ionizing radiation exposure can exploit these phenomenon to create a favorable target expression and location for delivery of other therapeutics or imaging probes.

We have shown in Figures 1–3 that galectin-1 is upregulated in three different human tumors and that galectin-1 expression can be selectively augmented by radiation exposure in endothelial cells. The uptake of galectin-1 specific isotope-labeled anginex peptide was shown to be high in tumors. This uptake further increased after radiation (Figure 1–3) in a manner that suggests that a ratio of 10-fold or greater galectin-1 expression between tumor endothelial cells and normal tissue may be obtained for targeting or imaging the tumor via the vasculature with our approach. The uptake of anginex conjugated fluorescent liposomes was also found to be increased significantly in the murine endothelial cell line (2H11) upon radiation exposure. This increase was not observed in the murine mammary SCK tumor cell line (Figures 4–5). Finally, the murine breast cancer co-culture model that we have developed also showed a similar trend of galectin-1 expression as observed *in vivo* (Figure 6). These studies have led us to infer that anginex is capable of serving as ligand for carrying conjugated drug loaded nanoparticles to the tumor vasculature associated receptor, galectin-1, especially with the use of radiation exposure as a means to specifically augment the targeting and/or as a byproduct of radiation exposure that occurs during normal radiotherapy.

The data presented in Figure 3 are an important aspect of our current findings. The standard dose of radiotherapy on a daily basis for patients is 2 Gy or higher. However, our data suggests that there may be reason to consider using very low doses of ionizing radiation to 'prime' the target in the tumor associated endothelium. In Figure 3, we performed a dose response with low doses, and found that after only 0.5 Gy (the lowest dose studied), the expression of galectin-1 increased as much or more than higher doses up to 4 Gy in a variety of endothelial cell types. We surmise from this data that a low dose of conformal radiation may improve the ability to target anginex-conjugated liposomes to the known tumor volume via the tumor vasculature for drug delivery or imaging contrast. In addition, the idea that a low dose of total body radiation could be used to stimulate the galectin-1 expression in not only the primary but other metastatic lesions that are known or unknown at the time of treatment. With this approach, highly effective systemic therapy may also be developed based on targeting only tumor associated endothelial cell Galectin-1 expression. Regardless, at the present time concurrent chemotherapy and radiation therapy are the standard of care for most types of cancers. Since the already elevated levels of galectin-1 can be further increased by radiation exposure the targeting of Galectin-1 protein, for tumor-specific drug delivery appears to be a promising strategy for the treatment of a wide variety of solid tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Elevated Galectin-1 expression in human tumors versus normal tissues and angine uptake in SCK tumors which is further augmented upon radiation exposure
(A) Expression profile of Galectin-1 in normal human and corresponding tumor tissues of the lung, bladder and breast was analyzed by western immunoblotting (ProSci, CA) with Actin as the loading control. The band intensity was quantified by densitometry using Image J software. **(B)** Biodistribution of [¹⁸F]-angine and its radiation induced uptake in SCK murine mammary carcinoma grown in nu/nu mice. A markedly increased uptake of [¹⁸F]-angine was observed in the SCK tumors as compared to the normal muscle tissue over the first 45 min post-i.p. injection. **(C)** Relative amount of radiation induced [¹⁸F]-angine uptake in 5 individual SCK tumors in the rear limb of nu/nu mice one day before and 2 h after local irradiation of the tumor with 2 Gy. SUV: Standard Uptake Value. The uptake of Angine in Animal 5 was 8 fold higher than baseline after radiation exposure.

Figure 2. Induction of Galectin-1 by radiation and its inhibition by anginex in BN Myeloma tumor implants in the rear limb of mice

(A) Immunohistochemical staining for Galectin-1 in human myeloma xenografts 48 h after untreated (control), 5 Gy, or treated with anginex (20 mg/kg/day) for 3 days and then irradiated with 5 Gy. and tissues harvested 48 h post-treatment. Images were scanned by Aperio Scanscope (B) The DAB stained positive areas in the images were quantified using Aperio Imagescope software. Competitive binding of anginex and Galectin-1 antibody to Galectin-1 or loss/death of Galectin-1 expressing cells caused reduced immunostaining of the tumor with the Galectin-1 antibody.

A **B** **C**

Figure 3. Radiation exposure induces the cell-surface expression of Galectin-1 in endothelial cells
(A) Expression profile of Galectin-1 at an increasing dose after 0.5–4 Gy of radiation exposure, with induction of Galectin-1 occurring at radiation dose of 0.5 Gy in the membranous fractions of various endothelial cell types [EA.hy926, Endothelial Progenitor cells (EPC) and HUVEC]. The human breast cancer cell line (MDA-MB231) however did not show any basal Galectin-1 expression in the membrane fraction or increase with radiation exposure; **(B)** Digital quantification of the results shown in A. **(C)** Galectin-1 expression in response to radiation in membranous fraction of murine 2H11 endothelial cells.

Figure 4. Anginex conjugated Fluorescent liposomes and their uptake by 2H11 murine endothelial cells

(A) Schematic of Anginex labeling strategy for Texas red labeled liposomes. (B) Increasing uptake of Anginex targeted liposomes by 2H11 murine endothelial cells with increasing concentration (left) and duration of incubation (right) of targeted liposomes. (C) Typical results of liposome uptake with non-targeted liposomes (NTL, Top) and targeted liposomes (TL, bottom) in murine 2H11 endothelial cells in culture.

Figure 5. Anginex tagged liposomal delivery to tumor and endothelial cells in culture before and after radiation exposure and competitive inhibition by Anginex or anti-galectin 1 antibody Uptake/binding of fluorescent liposomes by murine mammary SCK tumor cells and 2H11 endothelial cells assessed by flow cytometry (relative x-median fluorescence on Y-axis) (**A**) followed by the graphical representation of the values obtained (**B**). Anginex targeted liposomes (TL) were taken up by both cell types to a greater extent than non-targeted liposomes (NTL), and prior exposure to 2 and 4 Gy radiation enhanced anginex-tagged liposome binding selectively in endothelial cells. Uptake/binding of fluorescent liposomes by endothelial cells in the presence of free anginex and anti-galectin-1 antibody (**C**) followed by graphical representation of the values obtained (**D**). To block anginex-tagged liposome uptake, 2H11 endothelial cells were pre-incubated with anginex (10 μ M) or anti-galectin-1 antibody (4 μ g/ml). The radiation-induced uptake was specifically inhibited by free anginex or anti-galectin-1 antibody (3rd & 4th bar in 4 Gy treatments). No change in uptake of non-targeted liposomes was observed (data not shown).

Figure 6. Growth of tumor from tumor spheroids in dorsal skin fold window chamber implants and galectin-1 expression on radiation exposure

(A) (i) Phase contrast and (ii) Fluorescence images of 7 day GFP-4T1-2H11 tumor-endothelial spheroid grown in a hanging drop of medium. (B) Elevated galectin-1 expression in 4T1-2H11 tumor-endothelial cell spheroids when compared to 4T1 tumor cell spheroids alone which is further induced in response to radiation exposure. CD34 is the tumor endothelial cell marker and Actin serves as the loading control. (C) Galectin-1 expression in lysates from tumors originating from spheroids in dorsal skin fold window chamber implants of nude mice. (D) Induction of galectin-1 expression in sections from tumors originating from tumor-endothelial cell spheroids in rear limb of nude mice upon radiation exposure of 2 Gy.