Tumor Galectin-1 Mediates Tumor Growth and Metastasis through Regulation of T-Cell Apoptosis

Alice Banh1, Jing Zhang1, Hongbin Cao1, Donna M. Bouley2, Shirley Kwok3, Christina Kong3, Amato J. Giaccia1, Albert C. Koong1, and Quynh-Thu Le1

Abstract
Galectin-1 (Gal-1), a carbohydrate-binding protein whose secretion is enhanced by hypoxia, promotes tumor aggressiveness by promoting angiogenesis and T-cell apoptosis. However, the importance of tumor versus host Gal-1 in tumor progression is undefined. Here we offer evidence that implicates tumor Gal-1 and its modulation of T-cell immunity in progression. Comparing Gal-1-deficient mice as hosts for Lewis lung carcinoma cells where Gal-1 levels were preserved or knocked down, we found that tumor Gal-1 was more critical than host Gal-1 in promoting tumor growth and spontaneous metastasis. Enhanced growth and metastasis associated with Gal-1 related to its immunomodulatory function, insofar as the benefits of Gal-1 expression to Lewis lung carcinoma growth were abolished in immunodeficient mice. In contrast, angiogenesis, as assessed by microvessel density count, was similar between tumors with divergent Gal-1 levels when examined at a comparable size. Our findings establish that tumor rather than host Gal-1 is responsible for mediating tumor progression through intratumoral immunomodulation, with broad implications in developing novel targeting strategies for Gal-1 in cancer. Cancer Res; 71(13): 4423–31. ©2011 AACR

Introduction
Galectins are members of a large carbohydrate-binding lectins that share a highly conserved carbohydrate recognition domain (CRD) that is responsible for β-galactoside binding (1). Recently, several galectins, specifically Gal-1, -3, and -9, have been implicated to play a role in cancer progression (2). Although included in the same family, these 3 galectins are different from each other in size, tissue distribution, and function (3). Gal-1 is considered to be the "prototype" galectin that has a CRD and can form homodimers, whereas Gal-3 and -9 are part of different galectin subgroups (3, 4). Although the majority of Gal-1 is secreted into the extracellular matrix through an unconventional pathway, independent of the endoplasmic reticulum/Golgi route (4–7), it can also be found in the cytoplasm and the nucleus (2, 8). Gal-1 is expressed in many tumor types including astrocytoma, melanoma, and prostate, thyroid, colon, bladder, and ovary carcinomas (8, 9), and its expression correlates with tumor aggressiveness and metastasis (10–13). In lung cancer, increased Gal-1 expression is closely associated with larger tumors, more nodal metastasis, and lower overall survival (14).

Gal-1 is well known for its roles in modulating T-cell homeostasis, especially during development in the thymus and after stimulation at the periphery (15, 16). However, the exact mechanism by which this protein promotes tumor aggressiveness is not well elucidated. It has been suggested that Gal-1 is involved in various pathologic processes such as tumor cell proliferation (17), aggregation (18), adhesion (19), migration (10), cytoskeletal reorganization (20), and apoptosis (21, 22). More recently, 2 exciting new functions of Gal-1 in tumor cells have emerged. These include upregulation of tumor angiogenesis and promotion of tumor immune privilege by modulating intratumoral T-cell survival (23–25). Thijsen and colleagues have showed that an antiangiogenic peptide (anginex) binds to Gal-1 in endothelial cells and this interaction resulted in a reduction of endothelial cell proliferation and migration (24). Another study showed that tumor growth and in vivo angiogenesis are impaired in Gal-1 null mice, suggesting that angiogenesis is a major function of Gal-1 in tumor development (25). In contrast, Rubinstein and colleagues showed that the level of Gal-1 secretion in cultured supernatant correlated with the extent of tumor-induced T-cell death in both murine and human melanoma cells. Targeted inhibition of Gal-1 expression in vivo rendered the mice resistant to tumor challenge, which is a process that requires functional CD4+ and CD8+ T-cell response (23). These results suggest that Gal-1 contributes to tumor immune privilege by modulating survival of T-cell subsets.
We have previously found that Gal-1 secretion is significantly enhanced under hypoxia in tumor cells and its tissue expression could be used to assess treatment outcome in a large cohort of head and neck cancer patients. In addition, we noted an inverse relationship between tumor Gal-1 protein expression and the level of intratumoral T cells in these tumors, suggesting that Gal-1 is a negative regulator of T-cell activation and survival in human tumors (26). In this current study, we investigate the role of tumor versus host Gal-1 on tumor progression and metastasis. In addition, the contribution of immunomodulatory versus proangiogenic function of Gal-1 on tumor progression is evaluated. Lewis lung carcinoma (LLC) tumors with high (control) and low Gal-1 (shGal-1) expression were implanted into Gal-1 wild-type (WT) and Gal-1 null (Gal−/−) mice. We found that the expression of tumor Gal-1, rather than that of the host, was essential for tumor growth and spontaneous metastasis. Lung metastasis was only found in mice bearing Gal-1–expressing tumors and not in the mice bearing shGal-1 tumors. These results remained consistent even when all tumors were allowed to reach to a comparable size. The effect of Gal-1 on intratumoral T-cell apoptosis and angiogenesis was also examined. We showed that the effect of Gal-1 on tumor growth and metastasis is dependent on the host immune function and its proangiogenic effect is no longer evident when T- or B-cell functions are removed in immunodeficient mice.

Materials and Methods

Generation of stable shGal-1 cells

A pLKO plasmid with a short hairpin RNA (shRNA) sequence, CCGGCTCATACTTCAATCTTGGC TTCTCGAGAAAGCGAGGATTGAAGTGGTTTTTT, was used to generate shGal-1 lentivirus (ThermoScientific Open Biosystems). Another pLKO plasmid with a scrambled (Scr) sequence (Addgene Inc.), CCTAAGTTAAGTCCGCTTC GCTGACAGAG GCGACTTAACCTTAGG, was used as a negative control. Generation of Scr and shGal-1 lentiviral particles was carried out using the Trans-Lentiviral Packaging System (ThermoScientific Open Biosystems) following the manufacturer's protocol. Gal-1 expression is detected by Western blot analysis. Equal amounts of total protein were electrophoresed on 12.5% SDS polyacrylamide gel and incubated overnight at 4°C with goat anti-Gal-1 antibody (1:1,000 dilution; R&D Systems). Following this incubation, membranes were probed with the horseradish peroxidase (HRP)-conjugated anti-goat (1:10,000 dilution; Invitrogen) and ECL detection (Amersham Biosciences). The cell lysis buffer is used as a negative control, and recombinant mouse Gal-1 is used as a positive control (1 μg; R&D Systems). The membranes were also stained with mouse anti-β-actin antibody (1:1,000 dilution; Sigma-Aldrich) as loading control. The secondary detection for β-actin with HRP-conjugated anti-mouse (1:10,000 dilution; Invitrogen) followed the same procedures as mentioned above.

In addition, Scr cells and shGal-1 cells were plated in triplicates of 5,000 cells/plate and allowed to proliferate for 6 days; the cell numbers were quantified every 2 days.

Mouse tumor models

All animal procedures were approved by the Institutional Animal Care and Use Committee at Stanford University. Lgals1 null (Gal−/−) breeder mice were obtained from the Consortium for Functional Glycomics (Scripps Research Institute). C57BL/6 mice (Jackson Laboratory) were bred with Gal−/− to generate WT littermates. The genotype of the mice was determined by PCRs, using the antisense primer: AAACCTTCAGCCGGGAGAAGG; WT primer: GACCACCATCCCTACACCCCCAG and Gal-1 null primer: CTATCAGGACATAGCGTTGG. WT allele is indicated by 380-bp fragment, whereas a 280-bp fragment appears for the Gal−/− allele on a 2% ethidium bromide agrose gel.

The WT and Gal-1 null mice were inoculated subcutaneously with either 2 × 105 Scr cells or shGal-1 cells at 2 months of age. A total of 60 mice were used for this study: Gal-1 null/Scr (n = 15), Gal-1 null/shGal-1 (n = 15), WT/Scr (n = 15), and WT/shGal-1 (n = 15). The animals were euthanized when the tumor volume reached approximately 1.0 to 2.0 cm3 or 10% of the total body weight. Separate experiments were also carried out to collect tissues at earlier time points postimplantation. Two months old Nod.Cg-Prkdcscid Il2rg−/−/Sz [nonobese diabetic/severe combined immunodeficient (NOD-SCID); n = 15], B6.Cg-Foxn1nu/J (T cell−/−; n = 15), and B6.129S2-Igε1ε−/− (B cell−/−; n = 15) mice were purchased from Jackson Laboratory to study the effects of immunity on tumor growth. Seven animals from each mouse strain were inoculated subcutaneously with Scr control cells (1.5 × 105 cells), whereas 5 animals from each mouse strain were implanted with shGal-1 cells (1.5 × 105 cells). Animals were euthanized when the tumor volume reached approximately 1.0 to 2.0 cm3 or 10% of the total body weight.

Immunohistochemistry

Paraffin-embedded sections of tumor were deparaffinized and incubated at 4°C overnight with a goat anti-Gal-1 antibody (1:50 dilution; R&D Systems), rabbit anti-CD3 (1:100 dilution; Cell Marque), or rabbit anti-Ki67 (1:100 dilution; Cell Marque). Secondary detection was carried out using either an anti-goat or anti-rabbit peroxidase polymer detection kit (Vector Laboratories). The staining signal was detected by using the 3,3’-diaminobenzidine (DAB) substrate kit (Vector Laboratories). The sections were counter stained with hematoxylin (Sigma-Aldrich). Images were acquired by a Leica DM6000 B microscope (Leica Microsystems Inc.). The images of tumor sections were taken at 400× magnification.

Tumor samples collected at 22 days postimplantation, and after 25 days when tumors reached their maximum allowable volume (10% of body mass, ~1.0–2.0 cm3), they were stained for angiogenic marker CD31. Scr tumors from WT animals were also collected at an early time point (approximately day 14) when these tumors were of comparable size as that of...
shGal-1 tumors at day 22 (~0.35 cm³) and stained for CD31. Paraffin-embedded sections were deparaffinized and incubated at 4°C overnight with goat anti-CD31 (1:100 dilution; Santa Cruz Biotechnology Inc.), followed by secondary detection as described above. Images were taken at 100× and 200× magnification. Images of CD31 staining at 100× magnification were quantified using ImageJ software. A total of 4 visual fields of 1.26-mm² area from each tumor section were quantified.

Hematoxylin and eosin (Sigma-Aldrich) staining of paraffin lung sections was used to determine the number of lung metastases in each animal. A total of 3 lung sections per mouse lung sample were quantified at 50× magnification. Paraffin lung sections were also stained for Gal-1 as mentioned above. The staining signal was detected by using the 3-amin-9-ethylcarbazole (AEC) substrate kit (Vector Laboratories). The images of lung sections were taken at 100× magnification.

Frozen tissue sections were warmed to room temperature and fixed in acetone on ice for 20 minutes. After fixation, slides were washed in PBS solution. Slides were incubated at 4°C overnight with rat anti-CD4 or rat anti-CD8 (1:100 dilution; BD Biosciences). After overnight incubation, the slides were rinsed with PBS and then incubated with Alexa Fluor 594 anti-rat secondary antibody (1:200 dilution; Invitrogen) at room temperature for 1 hour. The labeled sections were fixed in 1% paraformaldehyde (PFA) solution for 15 minutes at room temperature, proceed by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) labeling with an in situ cell death detection kit (Roche Applied Science), using the method suggested by the manufacturer. All sections were mounted with medium containing 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Immunofluorescence images were acquired at 400× magnification, using a Leica TCS SP2 confocal microscope (Leica Microsystems Inc.). Total positive TUNEL nuclei and TUNEL-positive cells colocalized with CD4 and CD8 membrane staining were quantified with ImageJ software. Four visual fields at 400× magnification per tumor section were quantified.

In vitro T-cell study
Splenocytes of WT C57BL/6 animals were collected by mechanical disruption of the spleens (23), and T-cell isolation was carried out using the EasySep mouse T-cell enrichment kit (STEMCELL Technologies Inc.). Concanavalin A (ConA; 5 μg/mL; Sigma-Aldrich) was used in this study to activate cultured T cells. T cells were cultured in RPMI 1640 medium and divided into 4 treatment groups: control, recombinant mouse Gal-1 (rmGal-1:10 μg/mL; R&D Systems), ConA, and ConA + rmGal-1. After 48 hours of treatment, the cells were fixed in 1% PFA at room temperature for 20 minutes and permeabilized with 70% ethanol on ice for 30 minutes. The cells were labeled for TUNEL, using the Apo-Direct kit (BD Biosciences). The cells were analyzed with a BD LSR II flow cytometer (BD Biosciences). The fluorescence-activated cell-sorting (FACS) data were processed with Flowjo software (Tree Star Inc.).

The effects of Gal-1 on T-cell proliferation was detected through carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) labeling. Freshly isolated T cells were diluted to 1 × 10⁶ cells/mL and labeled with 1 μmol/L CFSE as suggested by the manufacturer’s protocol. CFSE-labeled T cells were divided into the 4 treatment groups and cultured under the same conditions as mentioned above. Following 48 hours of treatment, the T cells were collected and analyzed through FACS.

Hypoxprobe-1 (pimonidazole) detection
A separate group of WT (n = 5) and Gal-1 null (n = 5) mice were used to detect hypoxic regions and T-cell death in Scr and shGal-1 tumors. WT mice were inoculated subcutaneously with 2 × 10⁵ Scr cells, whereas Gal-1 null mice were inoculated with the same number of shGal-1 cells and the tumors were allowed to grow for 2 weeks. Intraperitoneal injection of hypoxprobe-1 (60 mg/kg; HPI Inc.) 90 minutes prior to euthanasia was used to detect hypoxic regions in the tumor.

Frozen tumor tissue sections were incubated at 4°C overnight with rat anti-CD4 or rat anti-CD8 as described above. After overnight incubation, the slides were rinsed with PBS and then incubated with Alexa Fluor 647 anti-rat secondary antibody (1:200 dilution; Invitrogen) at room temperature for 1 hour. All sections were also incubated with mouse anti-hypoxprobe-1 (1:50; HPI Inc.) and then incubated with Alexa Fluor 594 anti-mouse secondary antibody (1:200 dilution; Invitrogen). The labeled sections were fixed in 1% PFA solution for 15 minutes at room temperature, proceed by TUNEL labeling by the method suggested by the manufacturer. All sections were mounted with medium containing DAPI. Immunofluorescence images were acquired at 400× magnification, using a Leica confocal imaging system.

Statistical analyses
Data are expressed as mean ± SEM. Statistical analysis was carried out using IBM SPSS 19 software (SPSS Inc.). A 2-way ANOVA and post hoc Tukey’s test was used determine the significant differences of the tumor growth rates between mice strain and treatment groups. Lung metastases, TUNEL, and CD31 measurements was analyzed with the unpaired Student’s t test. A P value 0.05 or less is considered to be statistically significant.

Results
Tumor growth and spontaneous metastasis in the in vivo mouse tumor models
WT LLC cells were infected with either a lentiviral control scramble construct (Scr) or a shGal-1 construct to create a knockdown Gal-1 cell line (shGal-1; Supplementary Fig. S1A). To assess the effects of host Gal-1 in tumor growth and metastasis, Gal-1−/− mice were bred with C57BL/6 mice to generate Gal-1 WT littermates. The WT (380 bp) and knockout (280 bp) genotypes were confirmed by PCR analysis (Supplementary Fig. S1B). Scr and shGal-1 LLC cells were implanted in
the flank of either WT or Gal-1 null mice to form subcutaneous tumors. Four groups of mouse/tumor were generated: Gal-1 WT/Scr (high Gal-1 expression in both host and tumor), Gal-1 null/Scr (no host Gal-1 expression, high in tumor), Gal-1 WT/shGal-1 (normal host Gal-1 expression, low in tumor), and Gal-1 null/shGal-1 (low Gal-1 expression in both). The mice were sacrificed in 2 different cohorts. In the first cohort, all 4 groups were euthanized at the same time, around day 22 when the difference in tumor size between the groups was the largest. In the second cohort, the tumors were allowed to reach the maximum comparable volume of approximately 1.0 to 2.0 cm$^3$ for all 4 groups and then sacrificed. This means that 4 groups were sacrificed at different time points because of differential tumor growth rate.

Figure 1A shows that the rate of tumor growth is significantly delayed by knocking down tumor but not host Gal-1.
lung tissues (Supplementary Fig. S4). Our result showed that intense in the lung metastases than in the adjacent normal showed that Gal-1 protein expression is significantly more staining of the lung sections from WT mice bearing Scr tumors tissue (Supplementary Fig. S1C). Furthermore Gal-1 continued to maintain low level of Gal-1 expression in Gal-1 null mice. We also confirmed that shGal-1 cells developed on an average 4.1/C6 tumors (Fig. 1B).

Interestingly, tumor Gal-1 expression also influenced the development of spontaneous lung metastases when the tumors were grown in the flank of these mice, as quantified by light microscopy. In this experiment, the tumors in the 4 groups were allowed to reach the maximal allowable size (see above). The animals were then sacrificed and the lungs were examined for spontaneous metastasis. None of the mice inoculated with shGal-1 tumor cells developed lung metastasis (even when the tumor size was >1.0 cm³), whereas Scr cells developed on an average 4.1 ± 0.4 metastases/lung section in the WT mice and 2.3 ± 0.4 metastases/lung section in Gal-1 null mice. We also confirmed that shGal-1 cells continued to maintain low level of Gal-1 expression in vivo by immunoblotting and immunohistochemical staining of tumor tissue (Supplementary Fig. S1C). Furthermore Gal-1 staining of the lung sections from WT mice bearing Scr tumors showed that Gal-1 protein expression is significantly more intense in the lung metastases than in the adjacent normal lung tissues (Supplementary Fig. S4). Our result showed that reduction of Gal-1 protein in the tumor, but not the host, suppressed both tumor growth and distant metastasis.

**Mechanism of tumor Gal-1 on cell growth and metastasis**

**Immunomodulation via T-cell apoptosis.** Gal-1 has been shown to promote tumor growth through several mechanisms. One major mechanism focuses on its inhibitory effect of tumor immunosurveillance by promoting intratumoral T-cell apoptosis (23), blocking T-cell activation (27), and inhibiting secretion of proinflammatory cytokines. Another major mechanism invokes its proangiogenic effect (25). Here we aimed to elucidate some of these mechanisms in our model.

Like others, we found that exposing splenocytes to recombinant Gal-1 resulted in increased T-cell apoptosis (by TUNEL) and suppressed T-cell division (measured by CSFE), especially when these cells were activated by ConA (Supplementary Fig. S2). We then proceeded to examine tumor growth and spontaneous metastasis for both Scr and shGal-1 tumor cells in several immunocompromised mouse models, hence eliminating the immunomodulatory effect of Gal-1. These included Nod.Cg-Prkdcscid IL2rgtm1Wjl/SzJ (NOD-SCID; lacking B/T/macrophage and natural killer cell function), B6.Cg-Foxn1nu/J (lacking T-cell function), and B6.129S2-Ign-6tm1Cgn/J (B cell function) mice. As shown in Figure 2A, both Scr and shGal-1 tumors grew at the same rate in these immunocompromised mice. Likewise, the number of spontaneous lung metastases was similar for Scr and shGal-1 tumors (Fig. 2B; 19.8 ± 3.8 metastases for Scr vs. 17.4 ± 2.8 metastases for shGal-1). These data strongly indicated...
that the enhancing effect of Gal-1 on tumor growth and metastasis that was previously noted in immunocompetent mice was most likely due to its immunomodulatory function.

To support this observation, we stained the mouse tumors for CD3, a pan-T-cell marker. As shown in Figure 3A, there was more CD3 staining in the shGal-1 tumors than in the Scr tumors, suggesting less T-cell death. We then colocalized CD4 (helper T-cell marker) and CD8 (cytotoxic T-cell marker) with TUNEL and quantified the number of apoptotic T cells within the tumors. As expected, there was significantly more T-cell apoptosis in the Scr tumors than in shGal-1 tumors in the WT mice (Fig. 3B). The CD8⁺ T-cell death in the Scr tumors (n = 4, 61.2 ± 8.3%) was significantly greater than that in shGal-1 tumors (n = 4, 15.3 ± 4.6%). Similarly, the CD4⁺ T-cell death in the Scr tumors (n = 4, 46.2 ± 7.5%) was higher than in shGal-1 tumors (n = 4, 3.9 ± 1.5%). Similar observation was made in tumors growing in Gal-1 null mice (Supplementary Fig. S3A).

Because hypoxia induces Gal-1 secretion, we hypothesize that we would observe more T-cell apoptosis in the hypoxic regions of Gal-1–expressing tumors but not in shGal-1 tumors. We colocalized CD4, CD8, TUNEL, and pimonidazole (a hypoxia marker) in the WT/Scr and Gal-1 null/shGal-1 tumors (Supplementary Fig. S5). Although we saw minimal T-cell apoptosis within the hypoxic regions of either tumors, we noted more viable CD4⁺ (Supplementary Fig. S5, top right) and CD8⁺ T cells (Supplementary Fig S5, bottom right) in the hypoxic areas of shGal-1 tumors than in Scr tumors. Moreover, Scr tumors had more apoptotic CD4⁺ and CD8⁺ T cells in the periphery of hypoxic regions. These data suggest that hypoxia enhances Gal-1 secretion and diffusion, which promotes T-cell apoptosis.

Effects of Gal-1 on angiogenesis. Several studies have indicated that Gal-1 also has a proangiogenic function, which may primarily drive tumor growth and metastasis (24, 25). Therefore, we evaluated the microvessel density (MVD) count by CD31 staining on tumor tissues collected at 3 different time points. For the first time point, both shGal-1 and Scr tumors were implanted at the same time (day 0) and sacrificed at day 22 when the tumor growth differential was the largest between the 2 tumor groups (n = 3/group). For the second time point, we sacrificed the mice when the tumors reached the maximum allowable volume (~1.5 cm³, n = 3/group). The time of sacrifice for the Scr group was around days 22 to 25 and for shGal-1 tumor was days 31 to 34. This time point assesses MVD when both tumors were of comparable large size. For the third time point, we sacrificed the mice bearing Scr tumors at a much earlier time when these tumors reached a volume similar to that of shGal-1 tumor at day 22 (approximately 0.3–0.4 cm³, n = 3/group). This time point assesses MVD when both tumors were of comparable small size. At the first time point, MVD was significantly lower in the shGal-1 tumors (34.0 ± 4.6 microvessels/mm²) than in Scr tumors (62.0 ± 5.7 microvessels/mm²) in WT host [Fig. 4A (I) and B (I)]. Similarly, lower MVD was noted in shGal-1 (27.0 ± 2.6 microvessels/mm²) than in Scr tumors (35.2 ± 3.7 microvessels/mm²) in Gal-1 null mice, but the difference was not as pronounced as in WT mice (Supplementary Fig. S3B, left panel). In contrast, similar MDVs were noted when shGal-1 and Scr tumors were of comparable large size [MVD: 40.9 ± 2.9 microvessels/mm² vs. 40.1 ± 2.6 microvessels/mm²; Fig. 4A (II) and B (II)] or comparable small size [MVD: 33.5 ± 1.6 microvessels/mm² vs. 32.7 ± 1.6 microvessels/mm²; Fig. 4A (III) and B (III)].

Figure 3. Silencing Gal-1 expression in tumor cells enhanced lymphocyte survival. A, CD3 staining (DAB detection; 200 × magnification; scale bar, 50 μm) showed greater intratumoral lymphocytes in the shGal-1 tumors than in the Scr tumors grown in the Gal-1 WT host. Staining of mouse lymph nodes is used as a positive control. B, colocalization of CD4 and CD8 (red) with TUNEL (green) was carried out on Gal-1 WT mouse tumors (400 × magnification; scale bar, 50 μm). Cell nucleus is labeled with DAPI (blue). The greatest amount of CD8 cell death is found in the Scr tumors (n = 4, 61.2 ± 8.3%) when compared with the shGal-1 tumors (n = 4, 15.3 ± 4.6%). Similarly, CD4 T-cell death in the Scr tumors (n = 4, 46.2 ± 7.5%) is greater than in the shGal-1 tumors (n = 4, 3.9 ± 1.5%).
Figure 4. Proangiogenic effect of Gal-1 is related to the tumor volume. A, quantification of the MVD count on the basis of CD31 staining was carried out on WT mouse tumor tissues (200× magnification; scale, 100 μm). The graph shows the time points when tumors were collected to examine for MVD. The MVD was examined at 22 days after tumor implantation when tumor volume between the Scr and shGal-1 tumors showed the largest difference (AI and BI). At 22 days postimplantation, the MVD was significantly reduced in the shGal-1 tumor (n = 3, 34.0 ± 4.6 microvessels/mm²; tumor volume: 0.35 ± 0.15 cm³) when compared with the Scr tumors (n = 3, 62.0 ± 5.7 microvessels/mm²; tumor volume: 0.85 ± 0.25 cm³). The MVD of tumors, collected 25 days after tumor implantation when tumor volume was comparable between the 2 groups, was also examined [A (II) and B (II)]. There was no difference in MVD between the Scr (n = 3, 40.1 ± 2.6 microvessels/mm²; tumor volume: 1.80 ± 0.25 cm³) and shGal-1 (n = 3, 40.9 ± 2.9 microvessels/mm²; tumor volume: 1.46 ± 0.42 cm³) tumors. Finally, the MVD of Scr tumors, collected when the tumor volume (0.36 ± 0.08 cm³, < 22 days) was comparable with that of shGal-1 tumor at day 22, was examined [A (III) and B (III)]. The MVD of the smaller Scr tumors (n = 3, 33.5 ± 1.6 microvessels/mm²) is similar to the MVD of shGal-1 at day 22 postimplantation (34.0 ± 4.6 microvessels/mm²). C, quantification of MVD in Scr and shGal-1 tumors grown in B-cell−/− and T-cell−/− mice showed no significant difference between the Scr and shGal-1 tumors. The average MVDs for Scr in the B-cell−/− host (n = 3, 43.3 ± 2.1 microvessels/mm²) and T-cell−/− host (n = 3, 22.8 ± 1.3 microvessels/mm²) did not differ from their respective shGal-1 tumors in the B-cell−/− host (n = 3, 37.7 ± 3.7 microvessels/mm²) and T-cell−/− host (n = 3, 23.9 ± 1.6 microvessels/mm²).
improvement in microvessels/mm² vs. 34.0 ± 4.6 microvessels/mm²; Fig. 4A (III) and B (III)). Similar trend was noted when the MVD of Scr tumors (47.5 ± 2.9 microvessels/mm²) was compared with that of shGal-1 tumors (42.4 ± 6.3 microvessels/mm²) tumors in Gal-1 null hosts (Supplementary Fig. S3B, right panel; n = 3/group). Moreover, the MVD of tumors grown in immunocompromised mice (T cell–/– and B cell–/–) showed no significant difference between Scr and shGal-1 tumors (Fig. 4C). The average MVDs for Scr tumors in the B-cell–/– host (43.3 ± 2.1 microvessels/mm², n = 3) and T-cell–/– host (22.8 ± 1.3 microvessels/mm², n = 3) did not differ from their respective shGal-1 tumors in the B-cell–/– host (37.7 ± 3.7 microvessels/mm², n = 3) and T-cell–/– host (23.9 ± 1.0 microvessels/mm², n = 3). Note that the Scr and shGal-1 tumors showed a similar growth rate throughout the time of observation in these immunocompromised mice. These data argue that Gal-1 immunomodulatory function governs its effect on angiogenesis, which was no longer apparent when the immune function was removed in immunocompromised mice.

Discussion

Gal-1 is a secreted protein that is expressed by both tumor and host cells from various organs (28). Rabinovich and colleagues had previously validated that Gal-1 plays an immunoregulatory role through T-cell apoptosis, using cultured peripheral blood mononuclear cells (29). It has also been shown that abrogating Gal-1 expression in Hs683 glioblastoma cells and B16F10 melanoma cells increased the sensitivity to chemotherapeutic agents (30, 31). The role of host Gal-1 and tumor cell Gal-1 during tumor progression is yet to be determined.

Here we show that tumor Gal-1 is more critical in promoting tumor growth and metastasis and that host Gal-1 is less essential in our model. Depleting Gal-1 in the tumor resulted in a significant tumor growth delay and aborted the development of spontaneous lung metastasis in subcutaneously implanted tumors. Tumors expressing Gal-1 grew at the same rate and had similar numbers of lung metastases regardless of the host phenotype (Gal-1 WT and null mice), indicating that tumor Gal-1 is sufficient to promote tumor growth and distant spread in these mice. In contrast, a much smaller tumor growth delay effect was noted when host Gal-1 was removed and it was observed only when Gal-1 was downregulated in the tumor. Because presumably the same protein was produced and secreted by the tumor and the host cells, a possible explanation for our findings is the difference in the level of Gal-1 protein generated and secreted between the tumor and the host normal tissues. Appreciably higher expression of Gal-1 mRNA has been reported for human lung cancers than in matched normal lung tissues (32). Similarly, we noted a considerably stronger expression of Gal-1 protein in the lung metastases from the Scr tumors than in the adjacent normal lungs of WT mice (Supplementary Fig. S4). Moreover, our previous study has shown that hypoxia, which a common phenomenon in solid tumors, significantly enhanced the secretion of Gal-1 protein from tumor cells. Because hypoxia is not present in most normal tissues, hypoxia-induced Gal-1 secretion from the tumor may also contribute to the differential effect noted for tumor and host Gal-1. Finally, the activity of this protein is enhanced when it forms homodimers, which are more likely to occur when it is secreted at high levels.

Several strategies presently exist for targeting Gal-1 activity; these include blocking antibodies to deplete Gal-1 and to interfere with its ligand binding, natural polysaccharides, or synthetic glycodendrimers that compete with its glycan-binding ability or the peptide anginex, which mainly binds to Gal-1 in endothelial cells (24, 33). If Gal-1 proangiogenic activity is most crucial for tumor growth and metastasis, then targeting Gal-1 with anginex may be better than other approaches, as it will be more specific with less normal tissue toxicity. On the other hand, if its immunomodulatory function is more crucial, then antibody or polysaccharide-targeting approach may be more effective. Here we showed that Gal-1 immunomodulatory function may be more critical than its proangiogenic function in our tumor model. In immunocompetent mice, there was significantly greater T-cell apoptosis for both CD4+ and CD8+ cells in Scr tumors than in shGal-1 tumors. This observation was consistent with previously published data for B16 melanoma cells (23). However, when either T- or B-cell functions are depleted, we no longer observed a difference in either the tumor growth rate or the MVD count between Scr and shGal-1 tumors. Together, these data suggest that Gal-1 immunomodulatory function governs its angiogenic effect and that targeting Gal-1 immunomodulatory property is an important avenue to explore for future therapy.

Interestingly, the growth rate for Scr and shGal-1 tumors when implanted in B-cell–/– mice shows no significant difference. Although immunomodulatory function of Gal-1 is primarily associated with its effects on T cells, it has also been shown to negatively regulate B-cell proliferation. Upon B-cell receptor activation, Gal-1 has been suggested to accelerate B-cell apoptosis via Bim upregulation (34–36). Moreover, past studies had shown that these B-cell–/– mice possess reduced numbers of CD4+ and CD8+ splenic population compared with WT animals (37). They have smaller memory CD4+ pool and their CD4+ cells produced less interleukin-2 (IL-2) upon antigen stimulation because of decreased CD4 expansion (38). These mice also failed to respond to T-cell–dependent ovalbumin stimulation, hence indicating abnormal T-cell function (37–39). Thus, the marginal T-cell function in these mice combined with effects of Gal-1 on B-cell function abolished the Gal-1 tumor growth–promoting effect in the B-cell–/– mice.

In summary, we show using genetic models that tumor Gal-1 is more essential than host Gal-1 in enhancing tumor growth and metastasis, which seems to be mediated via its immunomodulatory function. These findings have broad implications in developing novel targeting strategies for Gal-1 in cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
Grant Support

This study was supported by Public Health Service grant number P01-CA67166 (to Q-T. Le, A. Banh, H. Cao, D.M. Bouley, A.C. Koong, and A.J. Giaccia) awarded by the National Cancer Institute, Department of Health and Human Services.

References


www.aacrjournals.org Cancer Res; 71(13) July 1, 2011

Gal-1 Regulates Tumor Growth and T-Cell Death

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received November 18, 2010; revised March 28, 2011; accepted April 15, 2011; published OnlineFirst May 5, 2011.
Tumor Galectin-1 Mediates Tumor Growth and Metastasis through Regulation of T-Cell Apoptosis

Alice Banh, Jing Zhang, Hongbin Cao, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-10-4157

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2011/05/05/0008-5472.CAN-10-4157.DC1

Cited articles
This article cites 39 articles, 17 of which you can access for free at:
http://cancerres.aacrjournals.org/content/71/13/4423.full.html#ref-list-1

Citing articles
This article has been cited by 16 HighWire-hosted articles. Access the articles at:
/content/71/13/4423.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.