Molecular and Cellular Pathobiology

Tumor Cells Secrete Galectin-1 to Enhance Endothelial Cell Activity

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Abstract

Tumor angiogenesis is a key event in cancer progression. Here, we report that tumors can stimulate tumor angiogenesis by secretion of galectin-1. Tumor growth and tumor angiogenesis of different tumor models are hampered in galectin-1–null (gal-1$^{-/-}$) mice. However, tumor angiogenesis is less affected when tumor cells express and secrete high levels of galectin-1. Furthermore, tumor endothelial cells in gal-1$^{-/-}$ mice take up galectin-1 that is secreted by tumor cells. Uptake of galectin-1 by cultured endothelial cells specifically promotes H-Ras signaling to the Raf/mitogen-activated protein kinase/extracellular signal-regulated kinase (Erk) kinase (Mek)/Erk cascade and stimulates endothelial cell proliferation and migration. Moreover, the activation can be blocked by galectin-1 inhibition as evidenced by hampered membrane translocation of H-Ras.GTP and impaired Raf/Mek/Erk phosphorylation after treatment with the galectin-1–targeting angiogenesis inhibitor anginex. Altogether, these data identify galectin-1 as a proangiogenic factor. These findings have direct implications for current efforts on galectin-1–targeted cancer therapies. *Cancer Res; 70(15); 6216–24. ©2010 AACR.*

Introduction

To promote angiogenesis, tumors secrete growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) that induce endothelial cell activation in nearby vessels. In response, the endothelial cells embark on a multistep process that results in new blood vessel formation. Evidence is increasing that endothelial galectins play a critical role in the endothelial adaptations associated with tumor angiogenesis (1). Especially, galectin-1 seems to fulfill a central role in the tumor vasculature during cancer progression. Elevated galectin-1 expression has been reported in the vasculature of many human tumors, including colon (2), head and neck, lung (3), prostate (4), and oral (5) cancers. Moreover, activation of endothelial cells by tumor cell–derived

growth signals results in elevated galectin-1 expression (2, 6, 7) and translocation of the protein to the endothelial cell surface (2, 8).

We have recently shown that endothelial galectin-1 is directly implicated in proper cell function during tumor angiogenesis (7). Knockdown of galectin-1 expression in endothelial cells inhibits proliferation and migration, whereas galectin-1–null (gal-1$^{-/-}$) mice display hampered tumor growth due to decreased angiogenic activity (7). In addition, it has been shown that endothelial galectin-1 is important for cell adhesion to extracellular matrix components (5) and that galectin-1 inhibitors can prevent endothelial tube formation (9). Based on all these findings, it has been proposed that targeting endothelial galectin-1 might be a promising angiostatic cancer therapy (1). Indeed, we have shown that tumors in gal-1$^{-/-}$ mice do not respond to galectin-1–targeted antiangiogenesis therapy (7).

Recent studies also suggest a role for exogenous galectin-1 in endothelial cell function. Knockdown of galectin-1 expression in glioblastoma tumor cells reduced the number of tumor vessels following injection of the cells in mice (10). In addition, exogenous galectin-1 was described to act as an endothelial chemoattractant and to enhance the viability and adhesion of cultured endothelial cells (5). Because increased expression of the protein has been reported in different human cancers (11–13), we hypothesized that galectin-1 secreted by tumor cells could also stimulate tumor angiogenesis via activation of endothelial cells. In the current study, we provide for the first time evidence that tumor-derived galectin-1 can indeed act as a proangiogenesis factor. We show that tumor endothelial cells in gal-1$^{-/-}$ mice take up galectin-1 from galectin-1–secreting tumor cells. Furthermore, we provide evidence that exogenous galectin-1 specifically

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promotes endothelial H-Ras signaling toward the Raf/mitogen-activated protein kinase/extracellular signal-regulated kinase (Erk) kinase (Mek)/Erk pathway and that it stimulates endothelial cell migration and proliferation. Moreover, this activation can be blocked by galectin-1 inhibition, which has direct implications for current efforts on galectin-1–targeted cancer therapies.

Materials and Methods

Materials

Human recombinant galectin-1 and polyclonal antihuman galectin-1 antibody were kindly provided by Dr. L. Baum (University of California, Los Angeles, CA). The antisense galectin-1 expression vector (pcDNA6-lag1) and the B16F10 galectin-1 knockdown (B16F10^{kd}) cells were a kind gift of Dr. G. Rabinovich (Instituto de Biología y Medicina Experimental, Buenos Aires, Argentina).

Cell culture

Endothelial cells were cultured as described previously (14). For the endothelial cell assays, serum-rich medium was replaced with culture medium containing 2% serum 24 h before the start of the experiment. B16F10 cells were cultured in DMEM supplemented with 5% FCS and L-glutamine. TC-1 cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10% FCS, L-glutamine, and 20 nmol/L β-mercaptoethanol.

Galectin-1 ELISA

A 96-well plate was coated overnight at 4°C with mouse monoclonal anti–galectin-1 antibody (Invitrogen) and blocked with 1% bovine serum albumin/PBS for 1 hour at room temperature before applying culture medium for 1 hour at room temperature. Subsequently, wells were incubated with rabbit polyclonal anti–galectin-1 antibody for 1 hour followed by a 30-minute incubation with biotin-conjugated swine anti-rabbit IgG antibody. Finally, horseradish peroxidase (HRP)–conjugated streptavidin was applied for 30 minutes and staining reaction was initiated by adding TMB staining solution. Reaction was stopped by adding 1 volume of 1 mol/L H_2SO_4, and absorbance was measured at 450 nm. Between all the incubation steps, the wells were washed three times with 0.1% Tween 20/milliQ. Negative controls included omission of secondary antibody, tertiary antibody, and/or streptavidin^{HRP}. A standard curve with recombinant human galectin-1 was included as positive control.

Mouse tumor models

The F9 teratocarcinoma model has been described previously (7). For additional experiments, the original 129P3/J gal-1$^{-/-}$ mutant mice (15) were backcrossed to the C57Bl6 background. On day 0, animals were injected s.c. with ∼1 × 10^5 B16F10 cells or ∼1 × 10^6 TC-1 cells. Tumor volumes were measured throughout the experiment. Animals were given water and standard chow *ad libitum*, and they were kept on a 12-hour light/dark cycle.

For the isogenic tumor model systems, 500,000 mouse B16F10 melanoma cells [either wild-type (wt; ATCC CRL-6475) or gal-1^{kd} cells] were injected i.v. into the tail vein of the mice. Six-week-old wt C57Bl6 mice were purchased from Charles Rivers. Gal-1–knockout mice were generated previously and bred in our laboratory (Poirier and Robertson, Development 1993; Institut Jacques Monod, Paris, France). Lungs were collected after euthanasia of dying mice and fixed for 48 hours in 4% formalin for further H&E staining. The *in vivo* experiments described in the present study were performed on the basis of Authorization No. LA1230568 of the Animal Ethics Committee of the Federal Department of Health, Nutritional Safety, and the Environment (Belgium).

Proliferation, migration, and chorioallantoic membrane assays

Migration, proliferation, and chorioallantoic membrane (CAM) assays were done as described elsewhere (16). Within each proliferation experiment, sample conditions were included in triplicate and independent proliferation and migration experiments were performed at least three times. For the CAM assay, $n = 8$ eggs were used in each treatment group.

Generation of stable gal-1^{kd} cells

To generate stable EC-RF24 gal-1^{kd} cells, 1 million EC-RF24 cells were transfected with 1 μg of linearized pcDNA6-lag1 (17) or empty pcDNA6 (mock) by electroporation using the Amaxa Nucleofector system, following the manufacturer's protocol. Cells were cultured as described above in the presence of 5 μg/mL blasticidin for further *in vitro* experiments. Downregulation of expression was determined by fluorescence-activated cell sorting (FACS) analysis according standard procedures using the anti–galectin-1 polyclonal antibody. The mock-transfected cells were used for normalization and FACS experiments were done in triplicate.

Analysis of galectin-1 uptake

Labeling of galectin-1 with Oregon Green 488 (OG488) was achieved by incubation of human recombinant galectin-1 with OG488 succinimidyl ester for 20 hours at a molecular ratio of 1:2. Progression of labeling was monitored by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry according to standard procedures. This confirmed that galectin-1 was, on average, labeled with 2 OG488 molecules. OG488-labeled galectin-1 (gal-1^{OG488}) or unlabeled galectin-1 was subsequently added to human umbilical vein endothelial cells (HUVEC) cultured in 2% serum–containing medium at the indicated concentrations. After 3 or 24 hours, medium was replaced with fresh, galectin-1–free medium and fluorescent images were acquired and processed using Adobe Photoshop CS2 with a Leica inverted fluorescence microscope using a Leica 1.4Mpix DFC300FX color camera.

Immunohistochemistry

Immunohistochemical stainings for galectin-1, CD31/34, CD45$^+$ cells, CD8$^+$ cells, macrophages, and polymorphonuclear cells were done as described previously (2, 7).

Real-time PCR

Isolation of total RNA from snap-frozen human tumor specimens, subsequent cDNA synthesis, and real-time PCR were done as described previously (2, 18). The galectin-1 mRNA expression was normalized to β-actin, cyclophilin A, β_2-microglobulin, and hypoxanthine phosphoribosyltransferase-1.

H-RAS signaling

RF24 cells were plated in six-wells plates at a density of 250,000 per well and grown for 24 hours in the presence of 10% FCS. The cells were then washed with medium containing 2% FCS and incubated for 48 hours in this medium in the absence and presence of 0.1 μmol/L recombinant galectin-1. Cells were then lysed and proteins (30–50 μg) were subjected to SDS-PAGE followed by Western immunoblotting using anti-pan Ras, anti–p-cRaf-1, anti-Raf, anti–p-Mek1/2 (Cell Signaling), anti-MEK1/2, anti–p-Erk, and anti-Erk2 antibodies (19). Protein bands were visualized by incubation with HRP secondary antibodies and enhanced chemiluminescence. Immunoblots were quantified by densitometry EZ-Quant Gel 2.1 software.

Statistics

All data are presented as mean ± SEM unless indicated otherwise. For comparison of data with normal distribution, Student's t test was used. Data that were not normally distributed were analyzed using the Mann-Whitney U test. The log-rank test was used to determine statistical difference in overall survival. Two-way ANOVA with Bonferroni post hoc test was used in the tumor growth experiments. All statistical computations were done using SPSS 12.0.1, except for the two-way ANOVA, which was done with GraphPad Prism v3.00.

Results

Elevated galectin-1 expression in tumor cells has been reported to be associated with enhanced tumor progression (11–13). To study the potential role of tumor-derived galectin-1 in tumor progression *in vivo*, we backcrossed the original 129sv gal-1$^{-/-}$ mouse to the C57Bl6 background. This allowed us to apply two syngeneic tumor models (i.e., B16F10 melanoma cells and TC-1 lung carcinoma cells) that show high mRNA expression and increased galectin-1 protein secretion as compared with the previously described F9 teratocarcinoma model (Fig. 1A and B). Similar to the previously published data for the F9 model (7), both B16F10 cells and TC-1 cells showed a significant inhibition of tumor growth in the gal-1$^{-/-}$ mice as compared with wt mice (gal-1^{wt}; Fig. 1C). The average growth inhibition in these tumor models ranged from 67% (F9 tumors) to 59% (B16F10 tumors) and 42% (TC-1 tumors), whereas maximal inhibition was ~70% for all three tumor types. Because galectin-1 has been associated with tumor immune escape (17), we determined whether galectin-1 expression levels induced different antitumor immune responses in gal-1^{wt} and gal-1$^{-/-}$ mice.
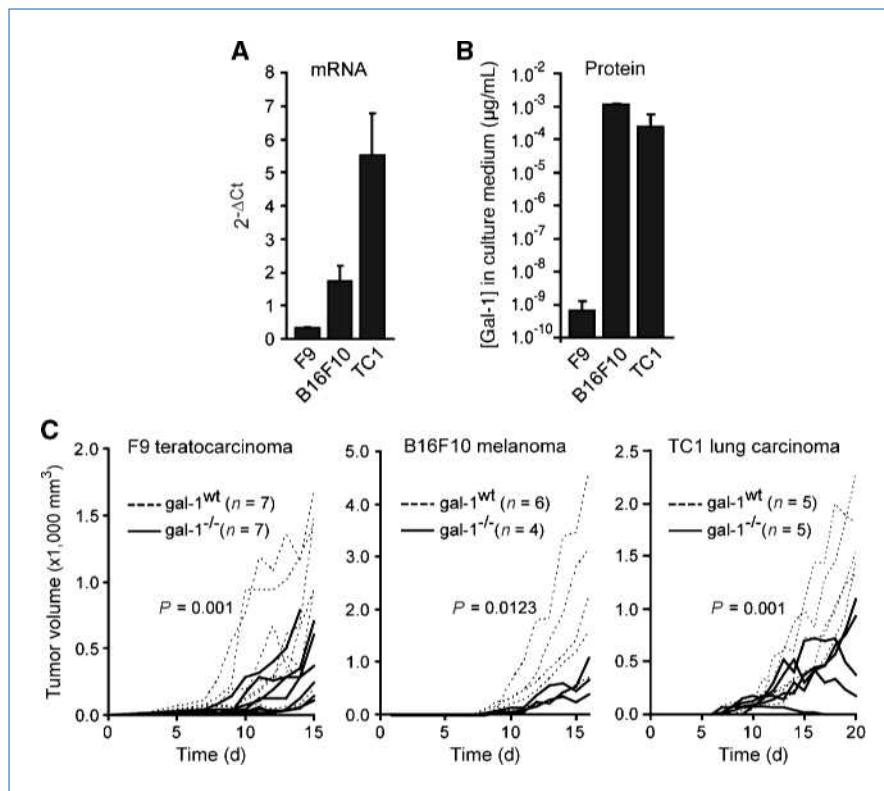

Figure 1. Impaired tumor progression in gal-1$^{-/-}$ mice is tumor type independent. A, galectin-1 mRNA expression levels in F9, B16F10, and TC-1 cells. B, galectin-1 protein levels in culture medium of F9, B16F10, and TC-1 cells as determined by ELISA. C, tumor growth curves of different tumor models in individual gal-1^{wt} mice (black dotted lines) or gal-1$^{-/-}$ mice (black lines). On day 0, mice were injected s.c. with F9 cells (1×10^6), B16F10 cells (1×10^5), or TC-1 cells (1×10^6).
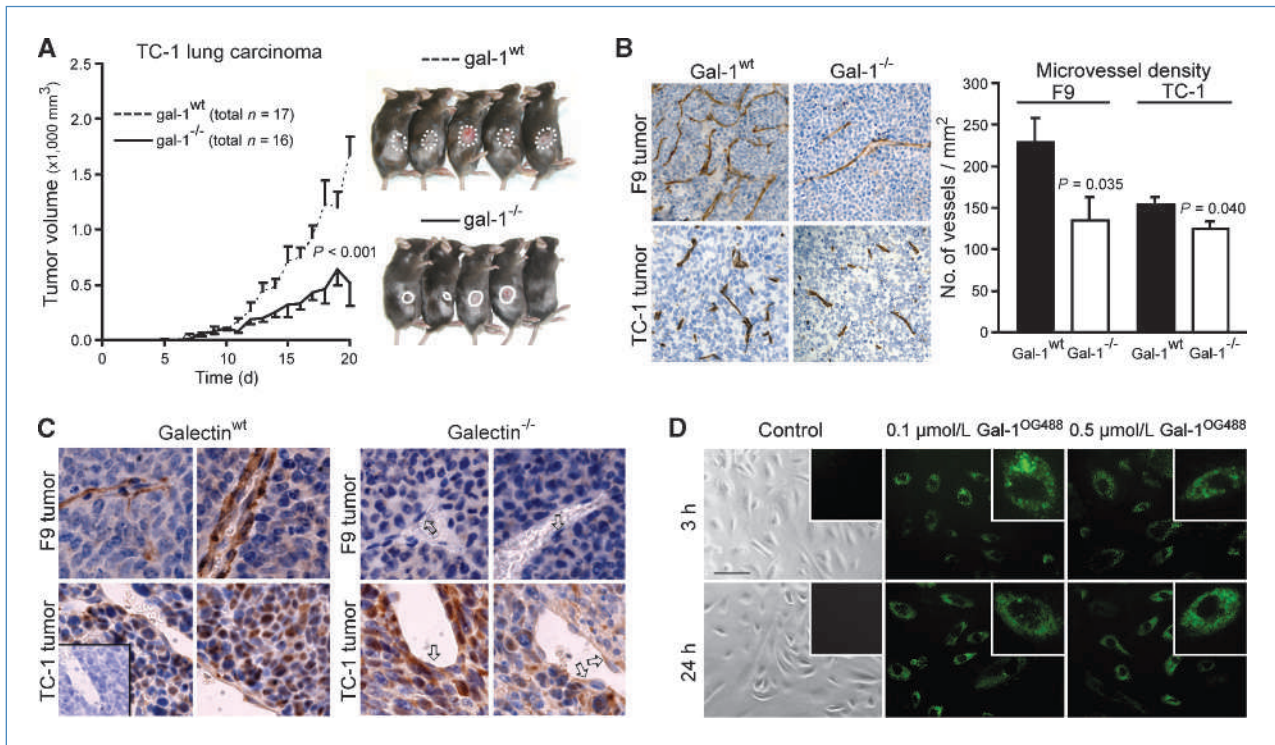
Figure 2. Tumor-derived galectin-1 can compensate for the lack of galectin-1 in the tumor endothelium of gal-1$^{-/-}$ mice. A, overall tumor growth of two independent experiments with TC-1 lung carcinoma cells in gal-1^{wt} and gal-1$^{-/-}$ mice. B, immunohistochemical vessel staining (brown) in TC-1 and F9 tumors in gal-1^{wt} and gal-1$^{-/-}$ mice. Diagram on the right shows the quantification of MVD in TC-1 and F9 tumors in gal-1^{wt} and gal-1$^{-/-}$ mice. C, immunohistochemical staining of galectin-1 (brown) in F9 and TC-1 tumors in gal-1^{wt} mice (left) and gal-1$^{-/-}$ mice (right). Arrows, endothelial cells that are negative for galectin-1 in the F9 tumors and positive for galectin-1 in the TC-1 tumors. D, fluorescent microscopic images of endothelial cells cultured in the presence of gal-1^{OG488} (green). Left, control cells treated with unlabeled galectin-1.

In all models, the tumor incidence and the onset of tumor growth were comparable between gal-1^{wt} and gal-1$^{-/-}$ mice (Supplementary Fig. S1). Furthermore, and as already reported for the F9 model (7), immunohistochemical quantification of tumor-infiltrating leukocytes revealed no significant differences in the numbers of CD45$^+$ cells, CD8$^+$ cells, macrophages, or polymorphonuclear cells between gal-1^{wt} and gal-1$^{-/-}$ mice in the B16F10 or the TC-1 tumors (Supplementary Fig. S1). This indicates that the immune response was comparable between gal-1^{wt} and gal-1$^{-/-}$ mice.

Because we have previously shown that tumor angiogenesis is impaired in gal-1$^{-/-}$ mice (7), we next investigated the involvement of tumor-derived galectin-1 in this process. We focused on the TC-1 model because these cells display an almost 20-fold higher galectin-1 expression as compared with F9 cells. A second experiment using a larger group of mice confirmed the impaired TC-1 tumor growth in gal-1$^{-/-}$ mice (Fig. 2A). Immunohistochemical staining of the endothelial cells and subsequent vessel quantification revealed a significantly decreased microvessel density (MVD) in the tumors of gal-1$^{-/-}$ mice as compared with gal-1^{wt} animals (Fig. 2B). Interestingly, with an MVD reduction of 20%, the inhibitory effect on angiogenesis was only half of that observed in the F9 tumors. Based on this, we hypothesized that the high level of galectin-1 expression by TC-1 cells could partially compen-

sate for the absence of galectin-1 in the tumor endothelium. As expected from the mRNA expression levels, we observed higher galectin-1 protein expression in TC-1 tumors as compared with F9 tumors (Fig. 2C, left). More interestingly, the endothelial cells in the TC-1 tumors of gal-1$^{-/-}$ mice occasionally stained positive for galectin-1, which was never observed in the F9 tumors (Fig. 2C, right). Comparable observations were made using an isogenic B16F10 tumor model. For this, B16F10 tumor cells, either gal-1^{wt} or gal-1^{kd} (17), were injected i.v. in gal-1^{wt} and gal-1$^{-/-}$ mice resulting in lung metastases. Injection of B16F10^{kd} cells resulted in prolonged survival in both gal-1^{wt} and gal-1$^{-/-}$ mice as compared with injection of B16F10^{wt} cells (Supplementary Fig. S2A). Subsequent analysis of the MVD in the lung metastases showed a gradual decrease in MVD, going from high in wt tumors of wt mice to low in knockdown tumors of null mice (Supplementary Fig. S2B). Again, immunohistochemical analysis indicated that endothelial cells in the null mice occasionally stained positive for galectin-1 (data not shown). Because the endothelial cells of the gal-1$^{-/-}$ mice do not express galectin-1, these data suggest that both in the TC-1 and in the B16F10 tumor models, the endothelial cells take up tumor-derived galectin-1.

To study whether exogenous galectin-1 is actually taken up by endothelial cells, we added gal-1^{OG488} to cultured

endothelial cells. Already 3 hours after exposure, cellular uptake of gal-1^{OG488} in vesicle-like structures could be observed. The uptake increased with time (Fig. 2D) and could be competed for by addition of unlabeled galectin-1 (Supplementary Fig. S3). FACS analysis after addition of trypan blue to quench extracellular fluorescence showed that gal-1^{OG488} was indeed present in the cytoplasm of the cells (data not shown). These data show that endothelial cells are able to rapidly take up large amounts of galectin-1 from their environment.

To study the direct effects of exogenous galectin-1 on the different key steps of the angiogenesis cascade, primary isolated HUVEC as well as the endothelial cell line EC-RF24 were cultured under low-serum conditions and subjected

to a broad concentration range of soluble galectin-1. We observed a small but significant induction of HUVEC proliferation at 0.1 μmol/L galectin-1, which was reversed at higher concentrations (Fig. 3A, left diagram). In EC-RF24 cells, the stimulatory effect of galectin-1 was more pronounced with significant induction of proliferation up to 2.5 μmol/L (Fig. 3A, right diagram). We next determined the effects of exogenous galectin-1 on endothelial cell migration. Similar to endothelial cell proliferation, inactivation of endothelial cells by low-serum conditions resulted in a hampered endothelial cell migration (Fig. 3B). In both HUVEC and EC-RF24, addition of galectin-1 to the culture medium effectively stimulated cell migration to a level similar to that obtained with bFGF
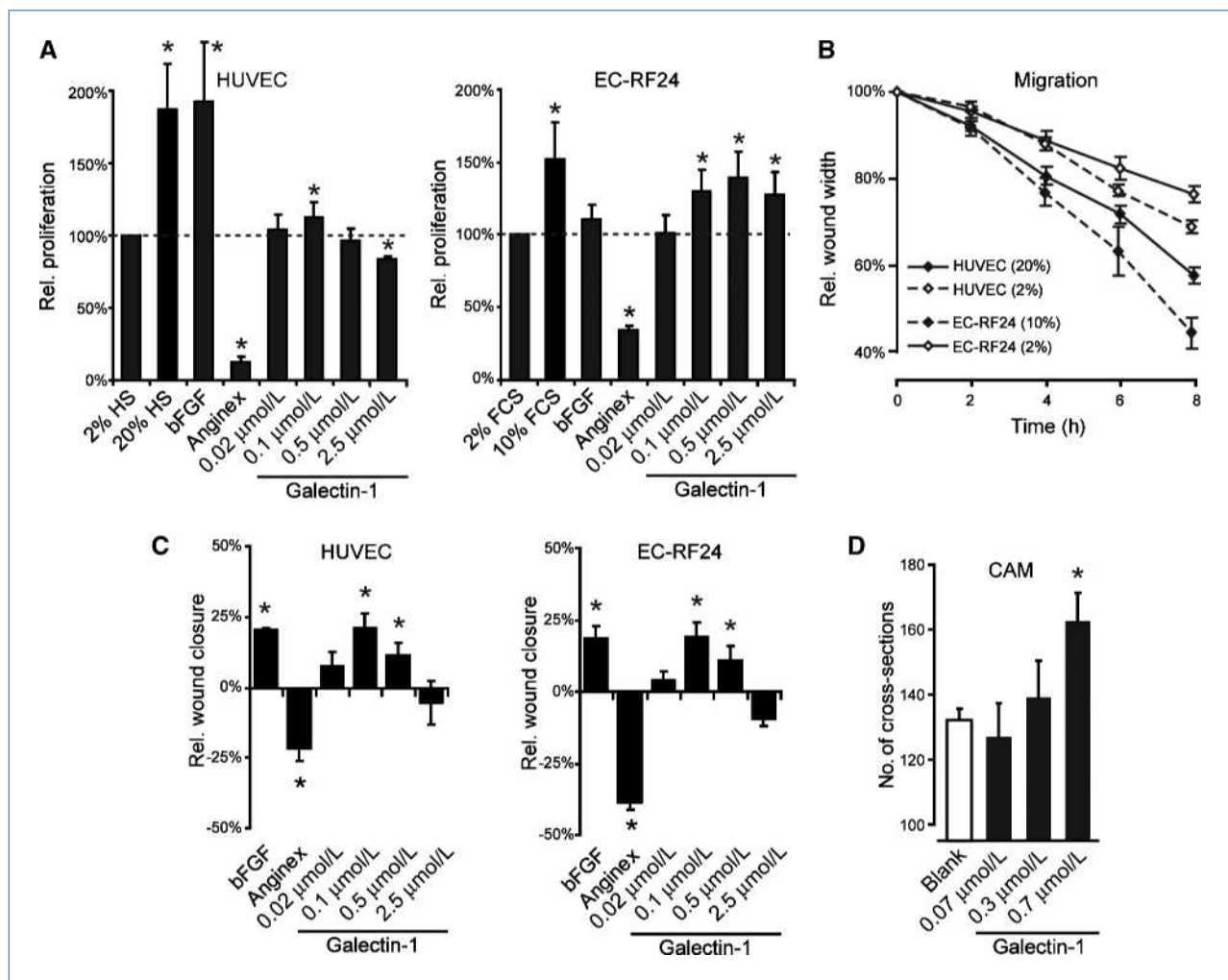

Figure 3. Exogenous galectin-1 induces endothelial cell proliferation and migration *in vitro* as well as angiogenesis *in vivo*. A, effect of exogenous galectin-1 on proliferation of HUVEC (left) or EC-RF24 (right) as determined by [^{3}H]thymidine incorporation. Cells were cultured for 3 d in 2% serum–containing medium in the presence of different stimuli. Proliferation is shown as relative values compared with inactivated cells (2% serum). B, migration of HUVEC and EC-RF24 as determined by the wound scratch assay. Confluent cell layers were cultured in medium containing 2% or 20% serum for 24 h, after which the wound was applied and wound width was measured every 2 h. Wound width is shown relative to the width at *t* = 0. C, effect of exogenous galectin-1 on migration of HUVEC (left) or EC-RF24 (right). Confluent cell layers were cultured in medium containing 2% serum for 24 h, after which the wound was applied and wound width was measured every 2 h in the presence of different stimuli. Migration at *t* = 8 h is shown as relative values compared with inactivated cells (2% serum). D, effect of exogenous galectin-1 on MVD in the *in ovo* chicken CAM assay. Galectin-1 was applied daily for 5 d in silicone rings placed on top of the CAM. Vessel density was determined as the number of vessel cross-sections with a predefined grid. *, P < 0.05.
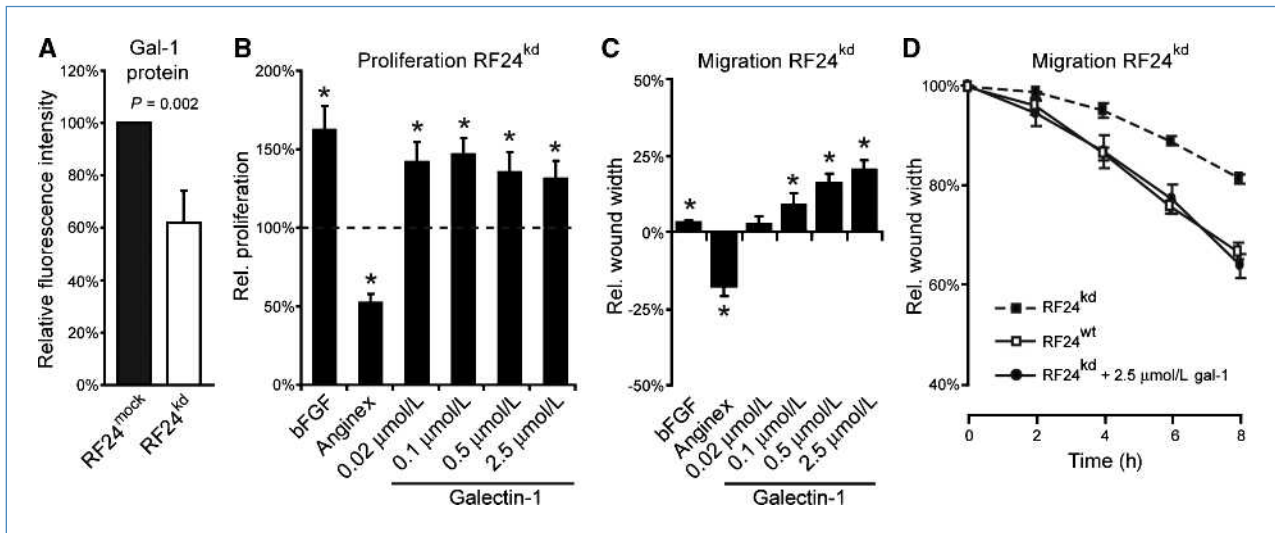
Figure 4. Uptake of exogenous galectin-1 by endothelial cells compensates for decreased levels of endogenous galectin-1. A, galectin-1 protein expression in mock-transfected or antisense galectin-1–transfected EC-RF24 cells. B, effect of exogenous galectin-1 on proliferation of RF24 knockdown (RF24^{kd}) cells. Cells were cultured for 3 d in 2% serum–containing medium in the presence of different stimuli. Proliferation is shown as relative values compared with inactivated cells (2% serum; dotted line). C, effect of exogenous galectin-1 on migration of RF24-knockdown cells. Confluent cell layers were cultured in medium containing 2% serum for 24 h, after which the wound was applied and wound width was measured every 2 h in the presence of different stimuli. Migration at $t = 8$ h is shown as relative values compared with inactivated cells (2% serum). *, $P < 0.05$. D, exogenous galectin-1 can fully restore the migration capacity of RF24^{kd} cells. Conditions are as in C, and migration at each time point is shown as relative values compared with $t = 0$.

(Fig. 3C). Experiments using the *in ovo* chicken CAM assay showed that exogenous galectin-1 induces a concentration-dependent increase in the number of vessels (Fig. 3D). These data show that exogenous galectin-1 exerts a proangiogenesis effect on endothelial cells. In nonendothelial cells, exogenous galectin-1 has been suggested to induce biphasic growth effects (20–22). We also observed biphasic effects on endothelial cell proliferation and migration. This suggests that the effects of exogenous galectin-1 are influenced by the cellular activation status. This was confirmed when increasing amounts of galectin-1 were added to cells cultured under high-serum conditions (Supplementary Fig. S4). Because we have previously shown that activated endothelial cells have increased galectin-1 levels, we questioned whether the biphasic response to exogenous galectin-1 was influenced by the endogenous endothelial galectin-1 expression. To study this, a stable knockdown cell line was generated by transfecting EC-RF24 cells with a previously described antisense galectin-1 expression construct (17). This induced a significant decrease in endogenous galectin-1 protein content (40% reduction, $P = 0.002$; Fig. 4A). As a result, both cell proliferation and migration were decreased as compared with wt and mock-transfected cells (data not shown). Adding increasing amounts of exogenous galectin-1 to EC-RF24 gal-1^{kd} cells compensated for the inhibitory effects on proliferation and migration without inducing any biphasic response (Fig. 4B and C). In fact, the decreased migratory activity of gal-1^{kd} cells was completely reversed by exogenously added 2.5 μmol/L galectin-1 (Fig. 4D). All these data show that exogenous galectin-1 can promote and facilitate angiogenesis by activation of endothelial cell function.

Finally, we set out to determine the mechanism underlying the galectin-induced stimulation of endothelial cells. An important intracellular function of galectin-1 involves the spatiotemporal organization of activated H-Ras during Ras signaling (23). We thus postulated that exogenous galectin-1 could promote endothelial cell proliferation and migration through enhanced H-Ras signaling. To test this, we examined whether exogenous galectin-1 affects Ras signaling to the Raf/Mek/Erk cascade in RF24 cells. Cells were treated for 48 hours with 0.1 μmol/L galectin-1 and the levels of active p-Raf, p-Mek, and p-Erk were determined by Western blotting (Fig. 5A, left). Subsequent quantification revealed a significant increase in Raf, Mek, and Erk phosphorylation (Fig. 5A, right), consistent with the notion that galectin-1 enhances H-Ras signaling in EC-RF24 cells. At higher galectin-1 concentrations, Ras signaling showed a similar biphasic response as observed in the proliferation assay (Supplementary Fig. S5).

Interestingly, the uptake of gal-1^{OG488}, as described above, occurred in small dot-like particles, resembling the H-Ras. GTP nanoclusters or rasosomes that are found at the plasma membrane and that occur as small, randomly moving cytoplasmic particles (24, 25). This also corroborates with previous observations that the galectin-1–binding angiostatic peptide anginex is taken up by endothelial cells and colocalizes with galectin-1 in dot-like vesicles (7). Indeed, anginex treatment of RF24 cells induced a decrease in Raf, Mek, and Erk phosphorylation (Fig. 5B), which was associated with membrane dislocation of activated H-Ras, as illustrated by treatment of RF24 cells that were transfected with GFP-tagged constitutively active H-Ras [GFP-H-Ras(G12V);
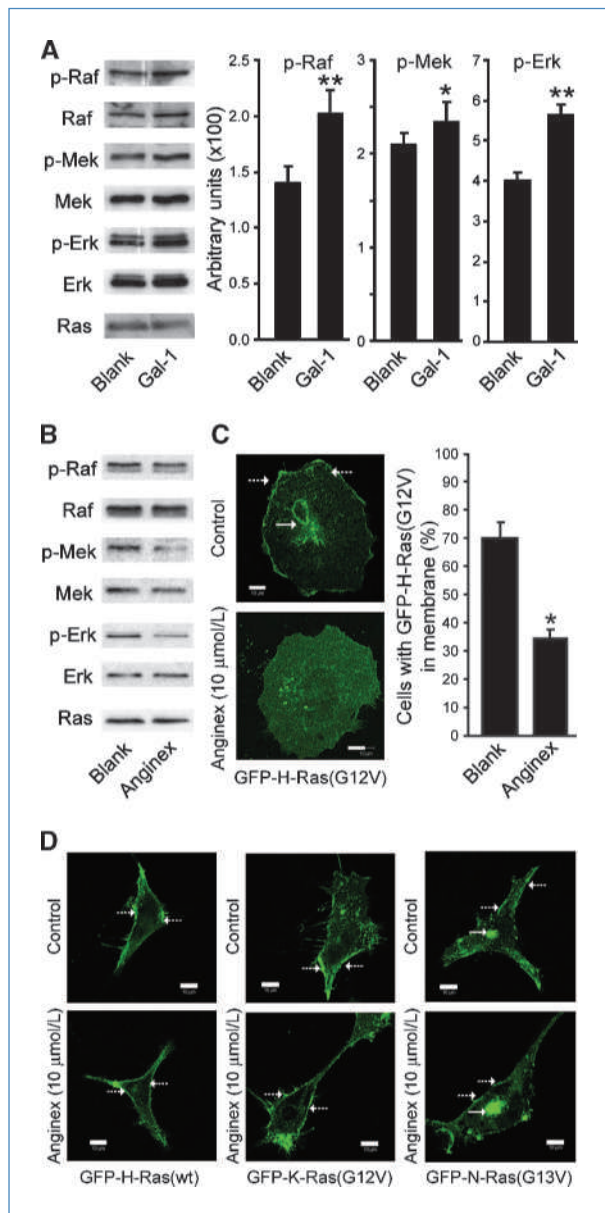
Figure 5. Effects of exogenous galectin-1 on H-Ras signaling in EC-RF24 cells. A, left, typical immunoblot of RF24 cell lysate after incubation with or without 0.1 μmol/L recombinant galectin-1. Diagrams on the right show densitometric quantification of different phosphorylated mitogen-activated protein kinases after treatment with or without 0.1 μmol/L recombinant galectin-1 ($n = 6$). *, $P < 0.05$; **, $P < 0.01$. B, typical immunoblot of RF24 cell lysate after incubation with or without 10 μmol/L anginex. C, typical fluorescence confocal microscopic images collected from control (top) and anginex-treated (bottom) RF24 cells transfected with GFP-H-Ras(G12V). Arrows, GFP-H-Ras(G12V) at the plasma membrane (dotted arrow) or in the Golgi (solid arrow). Bar, 10 μm. Diagram on the right shows the number of cells in which GFP-H-Ras is located in the plasma membrane, expressed as percentage of the total cell number. *, $P < 0.005$. D, typical fluorescence confocal microscopic images collected from control cells (top) and anginex-treated (bottom) RF24 cells transfected with GFP-H-Ras(wt) (left), GFP-K-Ras(G12V) (middle), and GFP-N-Ras(G13V) (right). Arrows are as described in C. Bar, 10 μm.

Fig. 5C, left]. Quantification revealed that galectin-1 inhibition induced a significant decrease in the percentage of cells with membrane-bound GFP-H-Ras(G12V) (Fig. 5C, right). Importantly, the plasma membrane localization of GFP-H-Ras (wt) was not affected (Fig. 5D, top left), which is consistent with the notion that galectin-1 specifically interacts with active H-Ras-GTP. This was further supported when we studied whether galectin-1 inhibition also induced displacement of the two other Ras isoforms (i.e., K-Ras and N-Ras) whose membrane localization is independent of galectin-1 (26). In untreated endothelial cells (Fig. 5D, top), GFP-K-Ras(G12V) was mainly localized to the plasma membrane (Fig. 5D, middle), whereas GFP-N-Ras(G13V) was localized to both the plasma membrane and the Golgi apparatus (Fig. 5D, right). This is in agreement with previous findings (27). Treatment with anginex did not induce a displacement of these Ras isoforms from their natural location (Fig. 5D, bottom). Altogether, these data suggest that exogenous galectin-1 can enhance endothelial activity via specific activation of H-Ras signaling.

Discussion

The current study describes a novel growth factor–like function for galectin-1 involving the promotion of tumor angiogenesis by enhancing the angiogenic activity of endothelial cells. We and others have previously shown that galectin-1 expression is increased in activated endothelial cells (1, 6, 7) and that endothelial activation increases exposure of the protein at the cell surface (2, 6). Increased galectin-1 expression has also been reported in the tumor endothelium of different human cancers (3–5, 7, 28). Thus, tumor-activated endothelial cells require high levels of galectin-1 for proper function during tumor angiogenesis. Our current data provide the first evidence that angiogenically activated endothelial cells can also derive the required galectin-1 from an external source (i.e., the tumor). We found that *in vitro* application of exogenous galectin-1 induces endothelial cell proliferation and migration, two key steps in the angiogenesis cascade. Hsieh and colleagues recently described that exogenous galectin-1 increases endothelial cell viability and adhesion and that galectin-1 can act as a chemoattractant for endothelial cells (5). Collectively, these data indicate that exogenous galectin-1 exerts a proangiogenesis effect on endothelial cells. Interestingly, exogenous galectin-1 has been previously described to have a biphasic effect on the growth of nonendothelial cells. Whereas low concentrations (nanomolar range) induce cell proliferation, high concentrations (micromolar range) of galectin-1 seem to have inhibitory effects (20–22). We also observed a biphasic effect of exogenous galectin-1 on HUVEC proliferation and migration. Moreover, in activated HUVEC, which already express high levels of endogenous galectin-1, an inhibitory effect was already noticeable at nanomolar concentrations. This suggests that the effects of exogenous galectin-1 are influenced by the cell activation status. Nevertheless, the *in ovo* CAM assay clearly showed an increased vessel density in response to exogenous galectin-1. Furthermore, our experiments in galectin-1–deficient mice show that tumor

angiogenesis in TC-1 tumors, which have high galectin-1 expression, is less affected by the absence of galectin-1 in the host vasculature as compared with F9 tumors, which have low galectin-1 expression. In the isogenic B16F10 tumor model, knockdown of galectin-1 in the tumor cells resulted in prolonged survival and lower MVD. A comparable observation was recently described for a glioblastoma tumor model where siRNA-induced inhibition of tumor galectin-1 expression resulted in a lower MVD (29). These data suggest that tumor-derived galectin-1 is involved in the regulation of tumor angiogenesis. In support of this, our current results obtained using gal-1^{kd} cells and gal-1$^{-/-}$ mice indicate that endothelial cells can indeed take up galectin-1 from their environment and use it to improve cell function. Altogether, these data suggest that tumor cells can secrete galectin-1 to promote tumor progression. Interestingly, galectin-1 is secreted by a nonclassic pathway, and there seem to exist common features between the secretion of galectin-1 and that of bFGF, another potent proangiogenesis growth factor (30–32). Moreover, Le and colleagues found a correlation between the expression of galectin-1 and the hypoxia marker CA-IX (33, 34). They also observed increased galectin-1 expression when different cancer cell lines were subjected to hypoxia (33, 34). It is thus tempting to speculate that the angiogenesis switch that tumor cells undergo in hypoxic conditions also involves upregulation of galectin-1 expression, and that the cells subsequently use a specific secretion route to shed galectin-1 and other angiogenesis-stimulating proteins into the environment.

Recently, it was suggested that exogenous galectin-1 facilitates NRP1/VEGFR2 signaling, which results in increased phosphorylation of Erk1 and c-jun NH$_2$-terminal kinase. However, blocking this pathway using NRP-1 targeting siRNA only marginally affected galectin-1–induced endothelial proliferation and Erk phosphorylation (5). Apparently, the role of galectin-1 in Ras signaling is not directly dependent on NRP1/VEGFR2 signaling. An important function of galectin-1 in Ras signaling involves the spatiotemporal organization of activated H-Ras (23). Galectin-1 mediates the formation of H-Ras.GTP/galectin-1 nanoclusters, which are the sites of activation of Raf/Mek/Erk (24, 35). In line with this, our data show increased phosphorylation of Raf, Mek, and Erk in endothelial cells after treatment with exogenous galectin-1. Moreover, the absorbed galectin-1 appeared in small dot-like particles, resembling the H-Ras.GTP nanoclusters or rasosomes, which are found at the plasma membrane and occur as small, randomly moving cytoplasmic particles (24, 25). This also corroborates with our previous observations that the galectin-1–binding angiostatic peptide anginex

is also taken up by endothelial cells and colocalizes with galectin-1 in dot-like vesicles (7). Thus, anginex is most likely shuttled into the endothelial cells by galectin-1 where it subsequently interferes with H-Ras nanocluster formation and downstream Ras signaling. Indeed, we found that anginex causes membrane dislocation of H-Ras.GTP and subsequent inhibition of Raf, Mek, and Erk phosphorylation. Altogether, these data favor a mechanism in which extracellular galectin-1 is taken up by the endothelial cells to facilitate the formation of H-Ras.GTP nanoclusters, which leads to enhanced Ras signaling and increased cell proliferation. How the endothelial cells take up galectin-1 remains to be elucidated. It is tempting to speculate that the mechanism responsible for the nonclassic secretion of the protein can also internalize the protein. A recent study in nonendothelial cells indicated that galectin-1 can be actively endocytosed. The uptake of galectin-1 was carbohydrate dependent and might involve GM1, a galectin-1–binding glycosphingolipid (36). Further analysis revealed that the internalization was facilitated by multiple routes (i.e., lipid-raft dependent and clathrin-mediated endocytosis; ref. 36). Additional studies are needed to determine whether such mechanisms are also involved in endothelial uptake of galectin-1.

In summary, the data presented in the current study provide evidence that tumor cells can induce and facilitate tumor angiogenesis by secreting galectin-1 into the environment. The secreted galectin-1 is used by the endothelial cells to enhance migration and proliferation. Furthermore, the endothelial cells take up galectin-1, which enhances H-Ras signaling. Together with its anti-inflammatory and prometastatic activities, this further exemplifies the importance of galectin-1 in cancer biology. Moreover, it identifies galactine-1 as an excellent target for combined cancer therapy because interfering with galectin-1 function might be beneficial at multiple processes involved in tumor progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Correction: Tumor Cells Secrete Galectin-1 to Enhance Endothelial Cell Activity

In this article (Cancer Res 2010;70:6216–24), which was published in the August 1, 2010, issue of *Cancer Research* (1), the *y*-axis of Fig. 4C was labeled incorrectly. Instead of "Rel. wound width", it should read "Rel. wound closure". The authors regret this error.

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Tumor Cells Secrete Galectin-1 to Enhance Endothelial Cell Activity

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