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# Galectin-3 Induces Endothelial Cell Morphogenesis and Angiogenesis

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**Increasing evidence suggests that carbohydrate-binding proteins play an essential role in tumor growth and metastasis. However, conflicting results on their function in the regulation of cell proliferation and differentiation during angiogenesis have been reported. We have examined the role of galectin-3 in the regulation of human umbilical vein endothelial cell proliferation, differentiation, migration, and neovascularization. Galectin-3, a carbohydrate-binding protein, with specificity for type 1 and 11 ABH blood group epitopes and polylactosamine glycan containing cell surface glycoproteins, is the major nonintegrin cellular laminin-binding protein. Because galectin-3 expression was shown to be associated in some tumor systems with metastasis, we questioned whether it induces endothelial cell morphogenesis. Here we show that galectin-3 affects chemotaxis and morphology and stimulates capillary tube formation of HUV-EC-C *in vitro* and angiogenesis *in vivo*. Endothelial cell morphogenesis is a carbohydrate-dependent process, as it is neutralized by specific sugars and antibodies. These findings demonstrate that endothelial cell surface carbohydrate recognition event(s) can induce a signaling cascade leading to the differentiation and angiogenesis of endothelial cells.** ***(Am J Pathol 2000, 156:899–909)***

Angiogenesis is a complex multistep process comprising a series of cellular events that lead to neovascularization from existing blood vessels and is associated with the process of inflammation, wound healing, tumor growth, and metastasis.[1–3] Determination of microvessel density in a growing cancer has prognostic value for recurrence and survival.[4]

In recent years numerous studies have focused on identifying angiogenesis stimulators, leading to the identification of several angiogenic factors. These can be divided into three groups of extracellular signals. The first comprises soluble growth molecules such as acid and basic fibroblast growth factors (aFGF and bFGF) and vascular endothelial growth factor (VEGF), which affect endothelial cell growth and differentiation.[5,6] The second group of factors inhibits proliferation and enhance differentiation of endothelial cells and includes transforming growth factor $\beta$ (TGF$\beta$),[7–9] angiogenin, as well as several low-molecular-weight substances.[10,11] The third group comprises extracellular matrix-bound cytokines released by proteolysis, which may contribute to angiogenic regulation.[11] Tumors can also generate inhibitors of angiogenesis, including angiostatin,[12,13] thrombospondin,[14,15] and endostatin.[16] In addition, a number of tumor-associated macrophages that secrete bFGF, tumor necrosis factor $\alpha$ (TNF-$\alpha$), endothelial growth factor (EGF), and VEGF, among other cytokines, were shown to play a role in tumor angiogenesis.[17,18] All of the above implies that angiogenesis is governed by a balance between positive and negative regulators within the microenvironment.[19,20] In addition to the above, it was shown that soluble forms of the carbohydrate-binding proteins (ie, E-selectin,[21,22] vascular cell adhesion molecule-1,[22] and P-selectin[23]) can promote endothelial cell migration and morphogenesis after binding to their respective glycoconjugate ligands. Antibodies directed against the carbohydrate epitopes of the ligand or E-selectin inhibited this process.[21,24] The clinical manifestation of elevated concentration of circulating E-selectin in sera of cancer patients[25–28] lends credence to these experimental observations. However, this premise was recently challenged by the finding that experimental angiogenesis can be induced normally in P- and E-selectin-deficient mice[29] and that endothelial cells from E-selectin-deficient mice undergo *in vitro* vascular tube formation.[30] Because endothelial cells' glycoproteins containing *N*-linked oligosaccharide structures are important for the tube formation process,[31] we questioned whether a different glycoprotein-binding protein, ie, galectin-3, can replace E-selectin and thus introduce an alternative signaling pathway into carbohydrate-mediated endothelial cell morphogenesis while bridging the above discrepancies.

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Galectin-3 is a member of a growing family of carbohydrate-binding proteins with molecular masses ranging from 29 to 34 kd. They share an affinity for β-galactoside-containing glycoconjugates and a conserved sequence of the sugar-binding motif (for reviews see refs.32–34). Galectin-3 (also known as Mac-2, CBP-35, ml-34, L-29, hL-31, and ϵBP) is a $M_r$ ~30,000 protein composed of two distinct structural motifs, an amino-terminal half containing Gly-X-Y tandem repeats characteristic of collagens and a carboxyl-terminal half containing the carbohydrate-binding site. Galectin-3 is expressed in a wide range of neoplasms and is involved in multiple biological processes through interaction with specific ligands, including cell growth, adhesion, differentiation, inflammation, apoptosis, and metastasis.[33,34] In human tumors, a direct relationship between galectin-3 levels and the stage of tumor progression in colon, gastric, thyroid, breast, and head and neck carcinomas has been demonstrated.[35–42]

It is obvious that the formation of new capillary vessels in a tumor is critical for its continuous growth and provides a gateway for the dissemination of malignant cells. We have previously transfected galectin-3 null human breast carcinoma BT-549 cells with human galectin-3 cDNA and established expressing cell clones that, unlike the parental cells, progressively grew and metastasized in nude mice.[38] This raised the question of a possible role for galectin-3 in angiogenesis.

We show here that soluble galectin-3 induces endothelial capillary tube formation *in vitro* and angiogenesis *in vivo*, and the specificity of the effect is established by antibodies and competitive sugars. In summary, these results suggest that angiogenesis could be mediated by carbohydrate recognition.

## Materials and Methods

### Cell Lines and Culture

Human umbilical vein endothelial cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD). The human breast cancer cell line BT-549 was a gift from Dr. Eric W. Thompson, and its galectin-3-transfected clone (11-9-1-4) was established as previously reported.[38] HUV-EC-C were cultured in Ham's F12K medium (Irvine Scientific, Irvine, CA) supplemented with 100 μg/ml heparin (Sigma Chemical Co., St. Louis, MO), 50 μg/ml endothelial cell growth supplement (Collaborative Biomedical Products, Bedford, MA), and 10% fetal calf serum (FCS) (Summit Biotechnology, Fort Collins, CO). BT-549 and 11-9-1-4 cells were maintained in Dulbecco's minimum essential medium (Gibco, Rockville, MD) containing 10% heat-inactivated FCS, essential and nonessential amino acids (Gibco), vitamins, and antibiotics (Mediatech Inc., Herndon, VA). The cells were maintained in a humidified chamber with 95% air and 5% $CO_2$ at 37°C. The HUV-EC-C were grown to confluence, detached from the monolayer with 0.25% trypsin, 2 mmol/L EDTA, and split 1:2; the breast cancer cell lines were split 1:4 for further cultivation.

For collection of the conditioned media, $4 \times 10^6$ human breast carcinoma cells were plated on a 60-mm tissue culture dish. The next day, the medium was replaced with serum-free medium after thorough washing with phosphate-buffered saline (PBS). The medium was collected 3 days later, concentrated 10-fold by centrifugation through ultrafree MC filter units (Millipore, Bedford, MA) with a 10,000-kd cut point, and analyzed for the presence of galectin-3 by Western blotting, using anti galectin-3 mAb.

### Recombinant Galectin-3, Antibodies, and Modified Citrus Pectin

Human recombinant galectin-3 was isolated and affinity purified as described.[43] Briefly, galectin-3 cDNA was expressed in *Escherichia coli* HMS174, using pET5a expression vector (Promega, Madison, WI). The protein was purified from the bacterial cell lysates, using an asialofetuin affinity column followed by extensive dialysis against PBS (pH 7.4). The pAb was prepared in rabbits against purified human recombinant galectin-3 (Genemed Biotechnologies, S. San Francisco, CA). The anti-galectin-3 mAb-producing hybridoma TIB-166 was purchased from ATCC. Mouse anti-β3-integrin monoclonal antibody was purchased from Transduction Laboratories (San Diego, CA). Anti-von Willebrand factor polyclonal antibody raised in rabbit was obtained from Sigma Chemical Co. Citrus pectin was purchased from Sigma Chemical Co. and modified into modified citrus pectin (MCP) as already described.[44]

### Capillary Tube Formation

To prepare Matrigel,[45] 200 μl of Matrigel (Collaborative Biomedical Products, MA) thawed on ice was added to each chamber of an eight-chamber slide. The air bubbles were carefully removed, and the slide was transferred to a 37°C incubator for 15 minutes. After gelation, $5 \times 10^4$ endothelial cells, which were separated from monolayers with trypsin treatment, were plated onto the gel in 200 μl medium. In some chambers, specific antibodies or PIS was added to the cells at the time of plating on Matrigel. The slides were incubated for 16 hours at 37°C. Alternatively, the Matrigel was diluted to 4.5 mg/ml with serum-free F12K medium and allowed to gel for 2 hours at 37°C. A total of $2 \times 10^4$ cells/200 μl medium were plated per chamber. Purified recombinant or secreted galectin-3 was added to some chambers. In some experiments galectin-3 was added with or without a competitive disaccharide (lactose), a competitive polysaccharide (MCP), and a noncompetitive disaccharide (sucrose), and endothelial cell migration and rearrangement was visualized after 4–6 hours.

### *Electrophoresis and Immunoblotting*

Endothelial cells were incubated with 10 μg/ml galectin-3 and lysed at different time intervals. The cell lysates or, in some experiments, conditioned media were suspended in reducing Tris-sodium dodecyl sulfate sample buffer, boiled for 5 minutes, and separated on a 8% or 12.5% polyacrylamide separating gel and 3.5% stacking gel. The separated proteins were then electroblotted to polyvinyl pyrrolidine fluoride (PVDF) membranes (MSI, Westborough, MA) and quenched in 5% nonfat dried milk in PBS for 4 hours, followed by incubation with the first antibody for 1 hour at room temperature. Subsequently, the membrane was washed five times with quench solution containing 0.1% Tween-20 and incubated with secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG or rabbit anti-rat IgG + IgM; Zymed, S. San Francisco, CA) for 1 hour and washed as above and processed for chemiluminescence.

### *Binding of Galectin-3 to HUV-EC-C and Scatchard Plot Analysis*

To determine the binding affinity and number of receptors on the endothelial cell surface, galectin-3 was iodinated in the presence of chloramine T. Briefly, 1 μg galectin-3 was incubated with 250 μCi $Na^{125}I$ in the presence of 40 μg chloramine T and 100 μl $H_2O$ on ice for 1 minute. The reaction was stopped by the addition of 20 μl of 1 mol/L KI. To remove the unbound labeled iodine, the reaction mixture was spun through Ultrafree-CL centrifugal filters (Millipore) precoated with 0.1% bovine serum albumin (BSA) in PBS. The labeled galectin-3 was resuspended in 50 μl of 0.1% BSA in 1× PBS and stored at 4°C for a maximum period of 1 month. One day before the assay HUV-EC-C were plated at a density of $5 \times 10^4$ cells per well in a 24-chamber plate. After washing, the wells were blocked with 0.1% BSA in 1× PBS for 30 minutes and incubated with 1–6 ng iodinated galectin-3/well in the presence or absence of 50 mmol/L lactose. After 2 hours of incubation at 4°C with constant shaking, the solution was removed and the cells were washed three times with 0.1% BSA-PBS. To measure the bound galectin-3, the cells were lysed with 1 mol/L NaOH for 30 minutes at room temperature, and the radioactivity was measured with a gamma counter.

For Scatchard plot analysis, the cells were incubated with 0.5 ng of iodinated galectin-3 in 0.1% BSA and 100 μl of 0.1% BSA containing a serial dilution of cold galectin-3 ranging from 0 to 50 ng for 2 hours at 4°C with constant shaking at 60 cycles/minute. The washes and counting were done as above. Values of ligand-receptor and [ligand receptor]/free ligand were calculated as follows:

Ligand receptor: [cold galectin-3(ng) + iodinated galectin-3 (ng)] × [bound galectin-3 (cpm)]/[Total counts added (cpm)]

Ligand receptor/free ligand: bound galectin-3 (cpm)/ total cpm − bound cpm

The affinity of the receptor ($K_D$ value) and receptor concentration ($R$ value) were determined using the method of Scatchard.[46]

### *Chemotaxis*

The assay was performed with a Boyden chamber. Briefly, 30 μl of conditioned medium from the galectin-3-secreting cells (11-9-1-4) and null galectin-3 cells (BT-549) and recombinant galectin-3 (0–20 μg/ml) in serum-free F12K medium were added to the lower chamber as a chemoattractant. HUV-EC-C ($5 \times 10^4$) were added to the upper chamber. The two chambers were separated with polycarbonate filters (8-μm pore size) and incubated at 37°C for 5 hours. At the end of the incubation period, the cells attached to the lower surface of the filter were fixed and stained using the Diff-Quik Stain set (Baxter, IL). A total of 10 fields were counted from each chamber under a microscope, and their sum was plotted. Each assay was carried out in quadruplicate.

### In Vivo *Angiogenesis*

Galectin-3-expressing (11-9-1-4) or nonexpressing (BT-549) cells[38] were injected subcutaneously into the dorsolateral region of nude mice (five per group) in the presence of Matrigel ($1 \times 10^6$ cells in 300 μl per mouse). After 10 days the tumors were removed along with the overlying skin, fixed with 10% buffered formalin, and processed for embedding, sectioning, and immunohistochemistry as described below.

NCR nude mice were also injected subcutaneously dorsolaterally with 0.4 ml Matrigel alone or in combination with 5 μg/ml bFGF or 10 μg/ml galectin-3 according to the method of Passaniti et al.[47] The injected Matrigel rapidly formed a solid gel that persisted for at least 10 days in mice. The mice were euthanized after 6 days, and the mass of Matrigel was removed along with overlying skin and fixed with 10% formaldehyde for at least 24 hours before it was embedded in paraffin. The paraffin blocks were then cut into 4-μm-thick sections and processed for immunohistochemistry.

### *Immunohistochemistry*

Immunohistochemistry was performed using a modification of the avidin-biotin peroxidase complex technique. Briefly, 4-μm tissue sections were deparaffinized, rehydrated, and placed in 3% hydrogen peroxide to inhibit endogenous peroxidase. The tissue sections were trypsinized with 0.1% trypsin and 0.1% $CaCl_2$ for 30 minutes at 37°C to expose the antigenic sites masked by formalin fixation, blocked for 1 hour with 3% normal goat serum (Sigma Chemical Co.), and subsequently incubated with antibody against von Willebrand factor for 60 minutes at a dilution of 1:1000. The sections were then treated with biotinylated secondary antibody (Vectastain

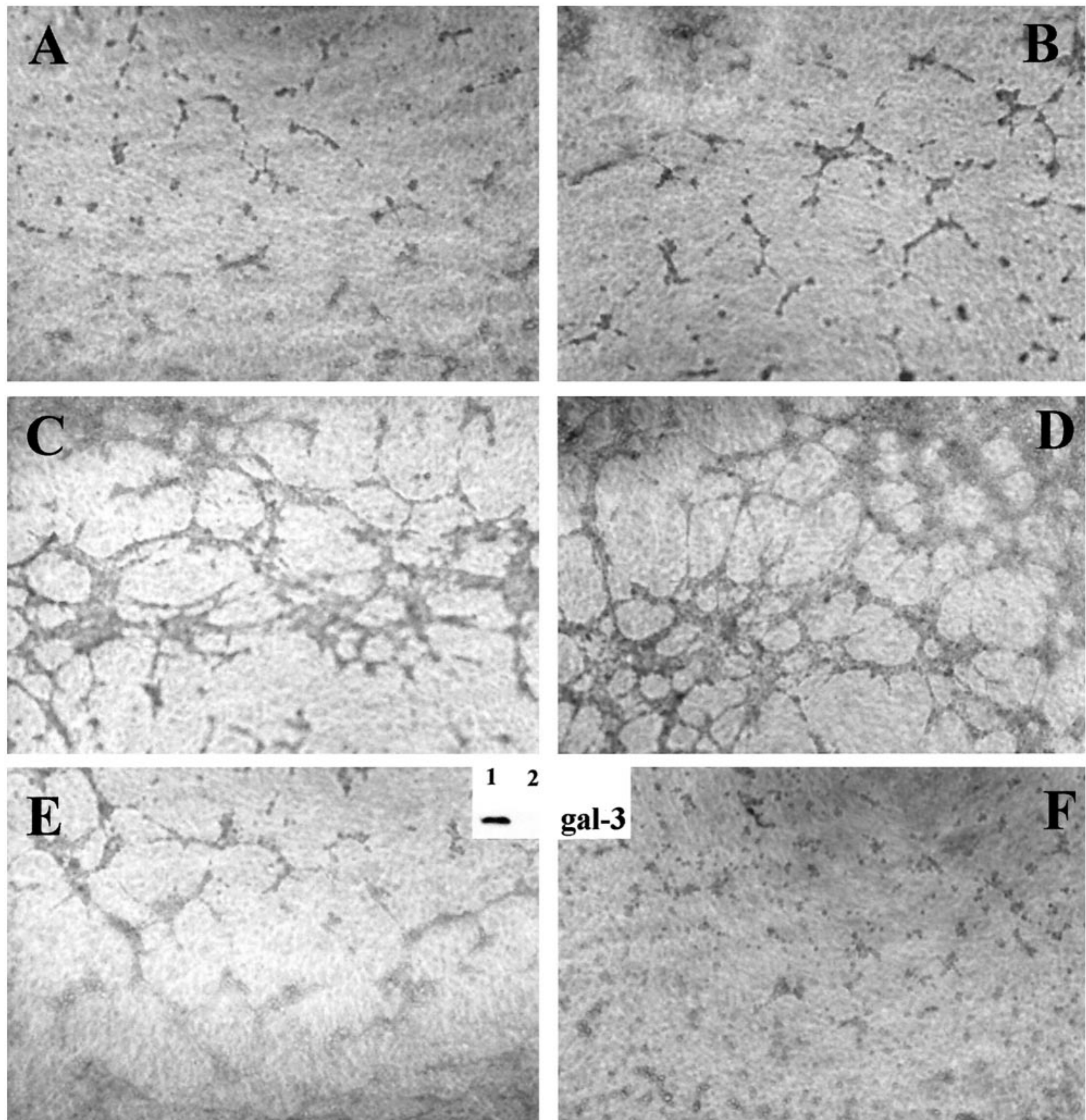


**Figure 1.** Endothelial cell organization on 4.5 mg/ml diluted Matrigel in the presence of exogenous galectin-3 (**A:** control; **B:** 1 μg/ml; **C:** 10 μg/ml; **D:** 20 μg/ml) or in the presence of conditioned medium from the galectin-3-secreting cell line 11-9-1-4 (**E**) and the null galectin-3 cell line BT-549 (**F**). **Inset:** Western blot of 100 μl conditioned medium. **Lane 1:** 11-9-1-4; **lane 2:** BT-549. Scale bar, 100 μm.

Elite ABC Kit; Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature, followed by avidin biotin complex reagent for 30 minutes and diaminobenzidine (Sigma Chemical Co) for 1 minute. Counterstaining was performed with hematoxylin.

For galectin-3 staining, basically a similar protocol was followed with a few modifications. The sections were not trypsinized, as formaldehyde fixation did not mask the antigenic sites of galectin-3. Blocking was performed with 3% normal rabbit serum. The primary antibody used was monoclonal antibody produced by TIB-166 hybridoma at a dilution of 1:2 for 60 minutes.

## Results

### Capillary Tube Formation on Matrigel

To establish the tube-forming property of endothelial cells on Matrigel, HUV-EC-C were plated on a gel formed by

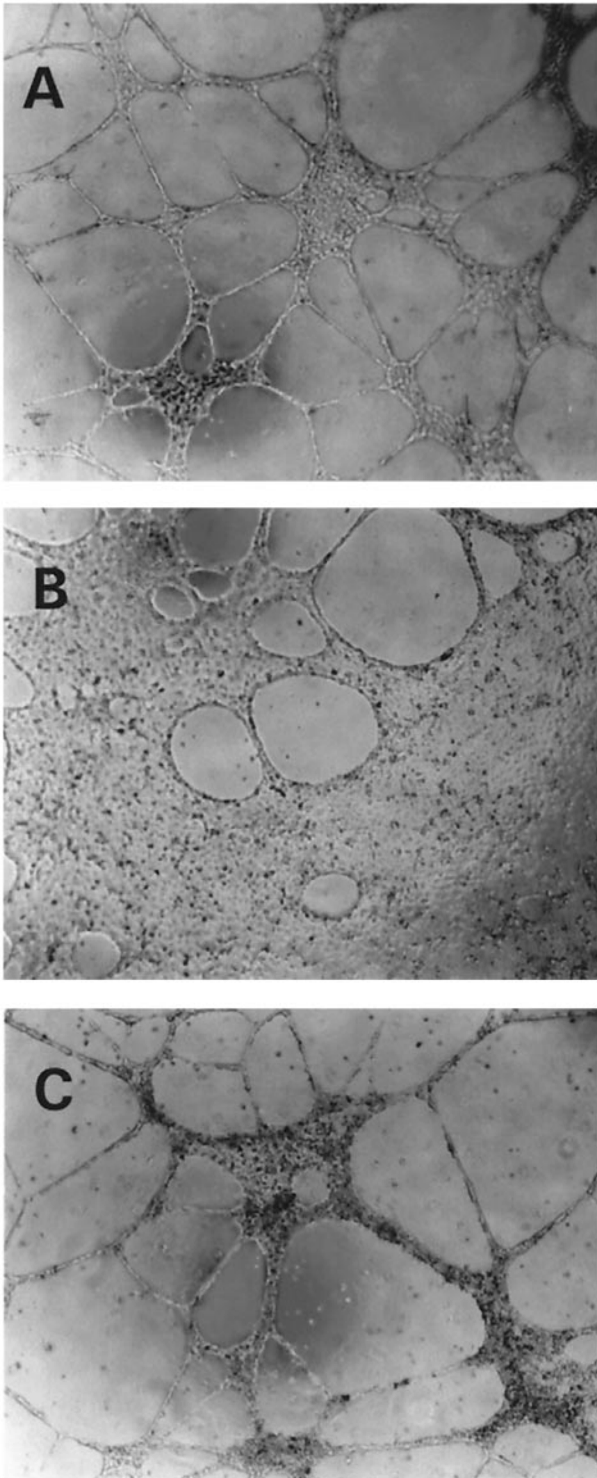


**Figure 2.** Capillary tube formation on Matrigel. **A:** Control; **B:** polyclonal antibody; **C:** preimmune serum. Scale bar, 100 μm.

200 μl of 14 mg/ml or 4.5 mg/ml Matrigel. On a gel prepared with undiluted Matrigel there was a rapid organization of endothelial cells into capillary-like structures (Figure 1A), whereas on diluted Matrigel, the cells underwent migration and organization into elongated structures without an interconnecting network (Figure 2A).

## *Stimulation of Capillary Tube Formation by Galectin-3*

To study the effect of galectin-3 on endothelial cell tube formation, we have analyzed the dose response of HUV-EC-C to soluble human galectin-3. Cells ($5 \times 10^4$) were plated on a gel formed by diluted Matrigel, in the presence of varying concentrations of galectin-3. Figure 1, A–D, show a concentration-dependent anastomosing network of endothelial cells. Similar stimulation of organization into a network was observed when HUV-EC-C were incubated with conditioned medium collected from the transfected human breast carcinoma 11-9-1-4 cells (Figure 1E), which secrete galectin-3 (inset: Lane 1). The conditioned medium collected from the galectin-3 null parental BT-549 cell culture (inset: Lane 2) failed to enhance the organization (Figure 1F), suggesting that this stimulation is not only restricted to the recombinant form but could also be mediated by cellular processed galectin-3. To further verify the importance of galectin-3, HUV-EC-C were plated on undiluted Matrigel in the presence and absence of a specific antibody or the preimmune serum. Figure 2, A–C, demonstrates that the basement-membrane-induced HUV-EC-C differentiation could be inhibited by anti-galectin-3 polyclonal antibody and not by preimmune serum control. There was a 50–90% reduction in the number of capillaries formed in the presence of pAb in various experiments. However, the mAb against the amino-terminal domain failed to inhibit tube formation (not shown).

Next, we questioned whether the effect of galectin-3 is mediated by its carbohydrate-binding domain; thus the effect of competitive sugars on tube formation was tested. We used MCP, a complex carbohydrate, and lactose, a disaccharide, which have been shown previously to compete with natural ligand (s) recognition by galectin-3.[44,48,49] HUV-EC-C were plated on a gel formed by Matrigel, in the presence of 0.1% MCP, a competitive polysaccharide; 50 mmol/L lactose, a competitive disaccharide; and 50 mmol/L sucrose, a noncompetitive disaccharide (Figure 3, B, C, and D, respectively). In the presence of MCP, cell motility and organization were completely inhibited (Figure 3B). The inhibitory effect was not due to cell damage and was reversible. In the presence of lactose, a 40–60% inhibition in tube-forming capability was observed (Figure 3C), whereas sucrose did not affect galectin-3-induced cell migration and organization (Figure 3D).

Next, we attempted to determine whether galectin-3 actually binds to HUV-EC-C. We found an increase in iodinated galectin-3 binding up to 3 ng/well, after which a slight decrease was observed (Figure 4). This binding was specific, as it was inhibited by 50 mmol/L lactose, indicating the involvement of carbohydrate recognition in the process and confirming the results shown in Figure 2B. Scatchard plot analysis (Figure 5) revealed the presence of two galectin-3 receptors on the HUV-EC-C cell surface: a high-affinity one with a $K_D$ of $0.537 \times 10^{-9}$ and a low-affinity one with a $K_D$ of $7.161 \times 10^{-9}$. The *R* values

**Figure 3.** Inhibition of capillary tube formation on Matrigel. **A:** Control; **B:** 0.1% modified citrus pectin; **C:** 50 mmol/L lactose; **D:** 50 mmol/L sucrose. Scale bar, 50 $\mu$m.

for the two receptors were $5.81 \times 10^3$ and $3.2 \times 10^4$, respectively.

## *Chemotactic Response of HUV-EC-C to Galectin-3*

Chemotaxis is an integral part of angiogenesis.[50] To establish whether galectin-3 stimulates the cell motility of

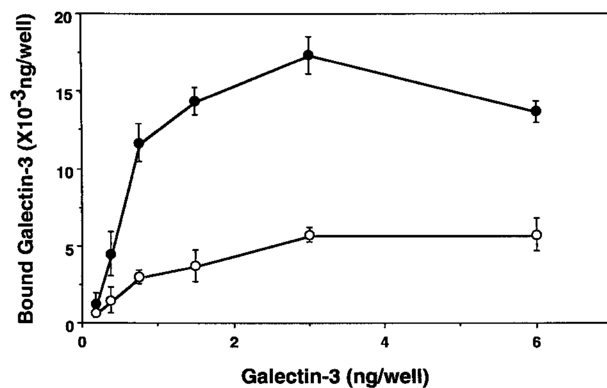


**Figure 4.** Binding assay of various concentrations of iodinated galectin-3 to HUV-EC-C in the presence (○) or absence (●) of 50 mmol/L lactose.

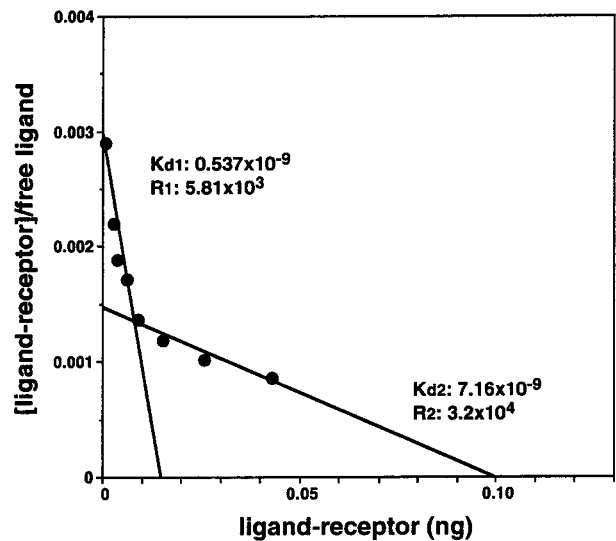


**Figure 5.** Scatchard plot analysis of $^{125}$I-labeled galectin-3 binding to the endothelial cell surface. Experiments were performed as described in Materials and Methods. Each value is represented as a mean of four determinations.

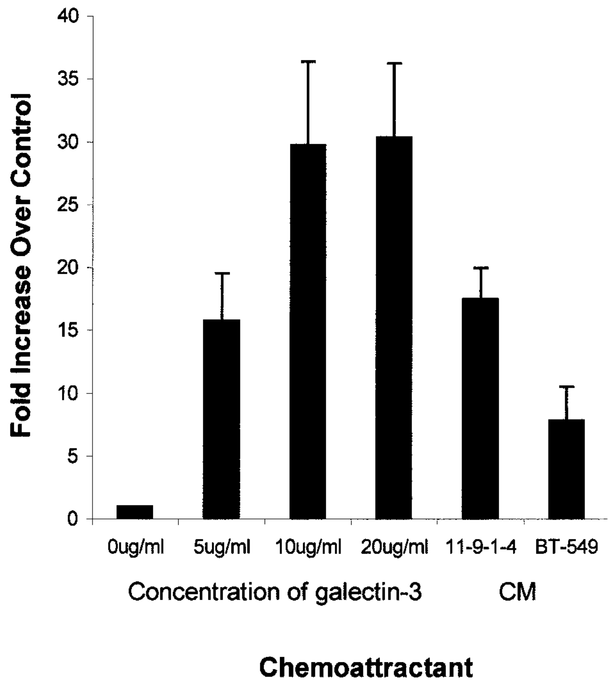


**Figure 6.** Chemotaxis of HUV-EC-C, by Boyden chamber assay. The bottom chamber contained 0–20 μg/ml galectin-3 in F12K, conditioned medium from galectin-3-secreting clone 11-9-1-4, or the conditioned medium from the null galectin-3 parental breast cancer cell line BT-549. An arbitrary unit of 1 was given to cells that migrated in 0 μg/ml gal-3. The rest of the values were calculated accordingly. Each point represents an average of eight readings.

HUV-EC-C by chemotaxis, like other carbohydrate-binding proteins,[22] we performed a Boyden chamber chemotaxis assay. Conditioned media containing galectin-3 (11-9-1-4 cells) or the conditioned medium devoid of it (BT-549 parental cells) or galectin-3 in different concentrations were added to the lower chamber as chemoattractants. The upper chamber contained the HUV-EC-C, and the two chambers were separated by a polycarbonate filter with 8-μm pores. After 5 hours of incubation the experiments were terminated and the cells that had migrated from the upper chamber through the filter toward the chemoattractants were counted. The results (Figure 6) show that only galectin-3 and conditioned medium of cells secreting galectin-3 induced a dose-dependent chemotactic response by the endothelial cells. *P* values were $<0.001$.

## In Vivo *Induction of Angiogenesis*

To test whether galectin-3 induces not only morphogenesis (*in vitro*) but also angiogenesis (*in vivo*), we injected the nude mice with galectin-3-expressing and nonexpressing cells, as well as recombinant galectin-3. A total of $1 \times 10^6$ BT-549 or 11-9-1-4 cells were injected subcutaneously into nude mice along with Matrigel. After 10 days the tumors were removed, embedded, sectioned, and stained with anti-von Willebrand factor for the presence of blood vessels (Figure 7, A and C). A serial

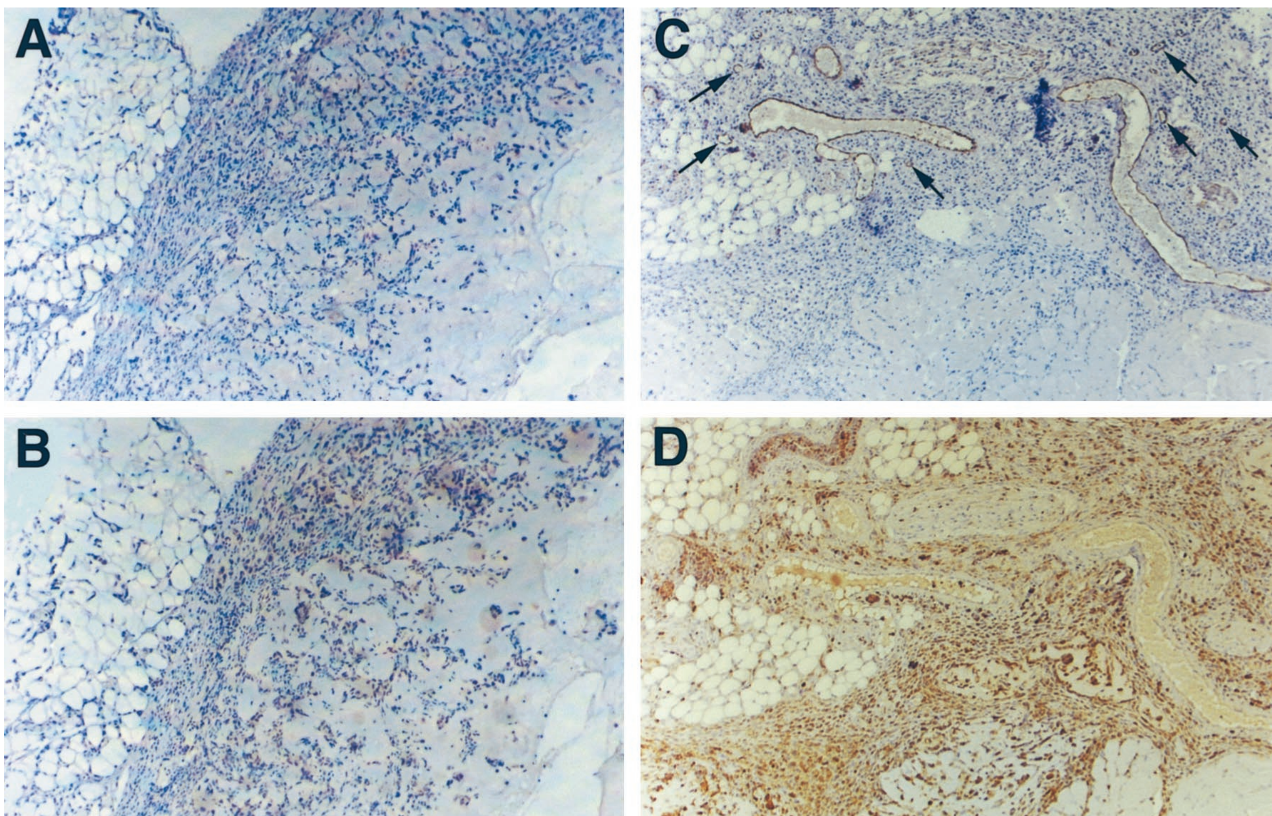


**Figure 7.** In vivo angiogenesis using BT-549 (**A** and **B**) and its galectin-3-transfected clone, 11-9-1-4 (**C** and **D**). Cells ($1 \times 10^6$) were injected with 300 μl Matrigel subcutaneously into nude mice. The tumors were removed after 10 days, fixed with 10% buffered formaldehyde, sectioned, and stained (brown) with anti-von Willebrand factor (**A** and **C**) and anti-galectin-3 antibodies (**B** and **D**). Scale bar, 8 μm.

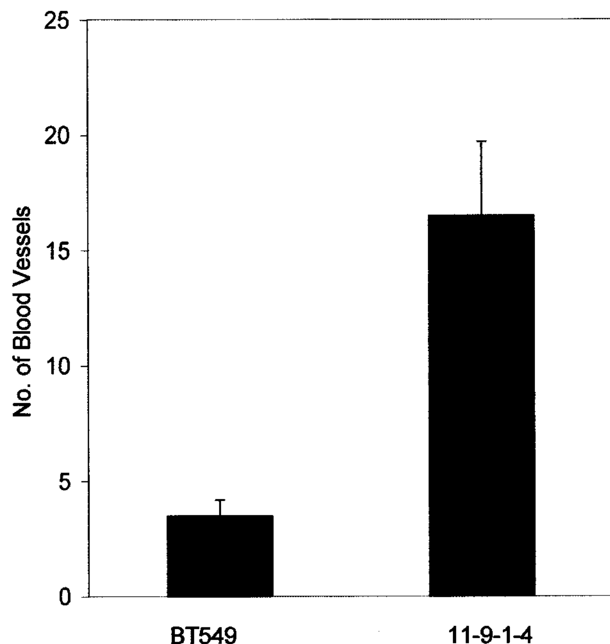


**Figure 8.** Microvessel density of Matrigel plugs injected with galectin-3-expressing and nonexpressing cells. The *P* value was 0.0038 as calculated by the Mann-Whitney test.

section was also immunostained for the presence of galectin-3 (Figure 7, B and D). Our results show the presence of tumor cells between the skin and the underlying muscle layer. In Figure 7, A and D, a few fat cells were visible at the basal layer of the skin. In the galectin-3-positive tumor (Figure 7, C and D), the fat cell layer was interrupted by tumor mass and blood vessels. At a similar position in the galectin-3-negative tumor (Figure 7, A and B), no blood vessels could be detected. Capillary density was calculated in the area immediately below the skin as the mean of the total number of vessels in five independent fields counted in three sections. In the tumor formed by BT-549 cells the capillary density was 3.3 ± 1.64 compared to 16.6 ± 7.86 in tumors formed by 11-9-1-4 cells (Figure 8). The *P* value was 0.0038, as calculated by the Mann-Whitney test.

To demonstrate whether galectin-3 by itself can induce neovascularization, we injected Matrigel supplemented with galectin-3 into the dorsolateral region of the nude mice.[47] When Matrigel is injected subcutaneously, it forms an insoluble gel capsule under the mouse skin and acts as a vehicle for the slow release of either galectin-3 or bFGF, which we used as a positive control. After 7 days the Matrigel was removed, embedded in paraffin, sectioned, and stained with antibodies against von Willebrand factor for the presence of neovessels. Matrigel alone did not induce any angiogenic or chemotactic response (Figure 9A). Matrigel supplemented with either galectin-3 or bFGF revealed an angiogenic reaction associated with the chemotactic response, as seen by the presence of cells infiltrating the gel (Figure 9, B and C, respectively). Microvessel density was 5.2 ± 2.3 and 6.5 ± 1.8 for galectin-3 and bFGF, respectively, thus supporting the notion that angiogenesis might be associated with an inflammatory response[51] and the conclusion that galectin-3 can act as an angiogenic factor.

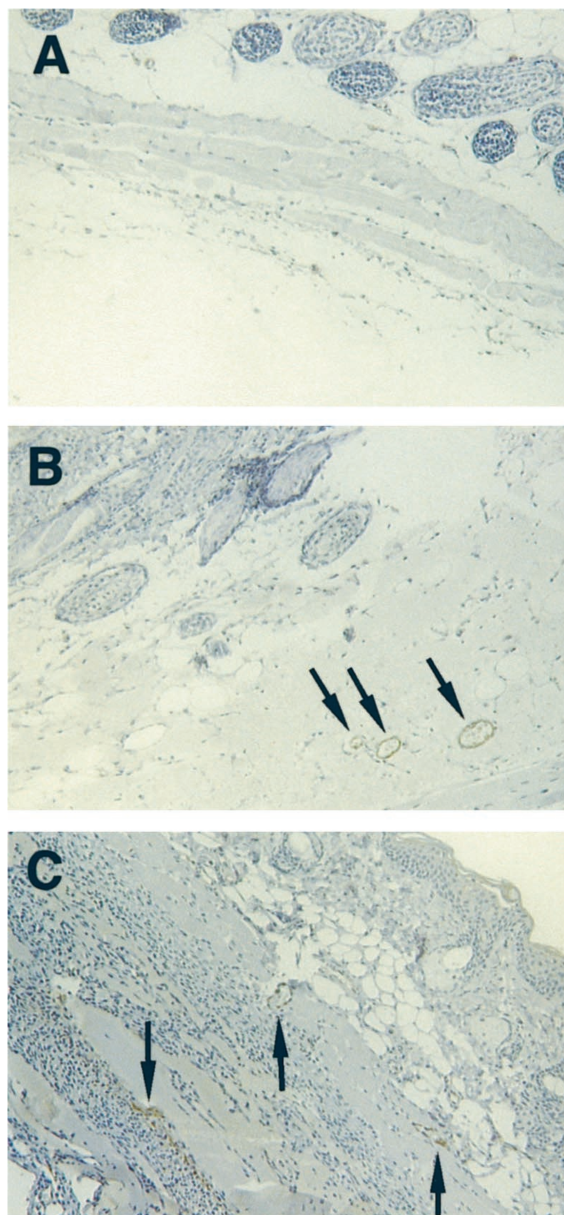


**Figure 9.** Factor VIII staining of neovessels. Four hundred microliters of Matrigel containing 5 μg/ml bFGF or 10 μg/ml galectin-3 was injected dorsolaterally into nude mice. Gels recovered after 7 days were sectioned and stained with von Willebrand factor antibody. **A:** Matrigel alone; **B:** with galectin-3; **C:** with bFGF. **Arrows** indicate blood vessels. M, Matrigel. Scale bar, 40 μm.

## Discussion

Tumor-associated angiogenesis is a hallmark of a growing tumor and thus serves as a rational target for cancer eradication. However, blocking the process of angiogenesis as an effective therapeutic modality still awaits the complete understanding of the biochemical and molecular signals that regulate the neovascularization process on one hand and the identification of inducers and inhib-

itors of angiogenesis on the other. Here we add galectin-3 to a growing list of angiogenesis-inducing proteins and highlight the role of carbohydrate recognition as a trigger signaling angiogenesis.

Galectins are a family of differentiation regulatory molecules that participate in a variety of biological functions, depending on the organ microenvironment and their specific cellular complementary glycoconjugates. Here we report a novel function for this carbohydrate-binding protein, showing that soluble recombinant galectin-3 or secreted galectin-3 stimulates *in vitro* and *in vivo* angiogenesis. Furthermore, this process is inhibited *in vitro* by specific antibodies and competitive sugar inhibitors.

Tumor-associated macrophages have been implicated at least in part in the mediation of neovascularization.[52,53] There is also a significant positive correlation between the number of macrophages in vascular tissues and angiogenesis.[53] Furthermore, many of the secreted products of activated macrophages have been characterized as inducers or inhibitors of angiogenesis.[18,54] It was shown previously that when monocytes differentiated into macrophages there was an induction of galectin-3 production and that galectin-3 could also be secreted by macrophages.[55–57] We presume that tumor-associated macrophages may also contribute to the pool of galectin-3 secreted by tumor cells into the extracellular matrix (ECM). In ECM galectin-3 may bind to one of its ligands. There are two known ligands of galectin-3 in the ECM, namely fibronectin and laminin.[58,59]

Galectin-3 secreted by tumor cells as well as macrophages may also act on endothelial cells to induce chemotaxis, facilitating their motility during the initial phase of tube formation, as we have shown in our results. Various angiogenic inducers selectively alter integrin profiles and influence cellular interactions with the ECM during neovascularization. It was shown that VEGF stimulates a type of neoangiogenesis in which endothelial cell migration and growth are dependent on attachment via $\alpha v\beta 5$ integrins, whereas neoangiogenesis induced by bFGF is dependent on $\alpha v\beta 3$ integrins (for a review see ref. 60). Similarly, TGF$\beta$ stimulates angiogenesis through up-regulation of $\alpha 2$, $\alpha 5$, and $\beta 1$ integrins.[61] Our results suggest a significant up-regulation of $\alpha v\beta 3$ integrins by treating the endothelial cells with exogenous galectin-3 (not shown). This could lead to cell motility in the initial phase of tube formation. The diffusible angiogenic factors can be placed in two categories. The factors in one category induce proliferation of endothelial cells *in vitro* and their differentiation (eg, bFGF, aFGF, VEGF, platelet-derived endothelial cell growth factor (PD-ECGF), and TGF$\beta$). The second category of factors induces differentiation but does not induce *in vitro* proliferation of endothelial cells (eg, angiogenin, TGF$\beta$, platelet-activating factor, soluble E-selectin, and TNF$\alpha$) (for a review see ref. 50). Galectin-3 appears to belong to the second category.

The formation of capillary tubes induced by galectin-3 was inhibited by the neutralization of the action of galectin-3 by its specific carbohydrate inhibitors or the specific antibodies. The effect of galectin-3 is comparable to that of other cell adhesion molecules (ie, VE-cadherin and E-selectin; for a review see ref. 62). Antibodies against VE-cadherin inhibited the capillary tube formation in fibrin or collagen gel over gel systems.[63] It was previously demonstrated that sialylated fucosylated N-linked oligosaccharides, potential ligands for soluble E selectin, were preferentially synthesized in endothelial cells undergoing tube formation. In addition, antibodies against E-selectin, sialyl Lewis X, and sialyl Lewis A inhibited tube formation.[22]

Similarly, bFGF, aFGF and many other cytokines bind to heparan sulfate proteoglycans via their sulfated oligosaccharide domains.[64–66] Inhibition of this binding interferes with the angiogenic phenomenon and the *in vivo* mitogenic response of the endothelial cells to this factor (for a review see ref. 66). These and the current observations, showing an inhibition of tube formation by neutralization of the carbohydrate recognition domain of galectin-3, indicate the importance of carbohydrate-protein interactions for angiogenesis. It is possible that after secretion, galectin-3 could either be stored in bound form to its ECM ligands or interact directly with endothelial cells. Scatchard plot analysis for the binding of galectin-3 to endothelial cells (Figure 5) demonstrates the presence of at least two cell surface receptors on EC. Inhibition of this binding by lactose indicates the involvement of the carbohydrate recognition domain in one or both of the receptor bindings. Once galectin-3 binds to its cell surface receptors, it may induce overexpression of integrin $\alpha v\beta 3$, leading to endothelial cell migration and attachment. Induction of *in vivo* angiogenesis by galectin-3-expressing cells or soluble galectin-3 further emphasizes the importance of carbohydrate-protein interactions during angiogenesis.

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## References

1. Folkman J: Angiogenesis in cancer, vascular rheumatoid and other diseases. Nature Med 1995, 1:27–31
2. Pluda JM: Tumor associated angiogenesis: mechanisms, clinical implications, and therapeutic strategies. Semin Oncol 1997, 24:203–218
3. Lee AH, Happerfield LC, Bobrow LG, Millis RR: Angiogenesis and inflammation in invasive carcinoma of the breast. J Clin Pathol 1997, 50:669–673
4. Vartanian RK, Weider N: Correlation of intratumoral endothelial cell proliferation with microvessel density, tumor angiogenesis, and tumor cell proliferation in breast carcinoma. Am J Pathol 1994, 144:1188–1194
5. Burgess WH, Macaig T: The heparin binding (fibroblast) growth factor family of proteins. Annu Rev Biochem 1989, 58:575–606
6. Conn G., Bayne M L, Soderman DD, Kwok PW, Sullivan KA, Palisi TM, Hope DA, Thomas KA: Amino acid and cDNA sequences of a vascular endothelial cell mitogen that is homologous to platelet derived growth factor. Proc Natl Acad Sci USA 1990, 87:2628–2632
7. Muller G, Behrens J, Nussbaumer U, Bohlen P, Birchmeier W: Inhibitory action of transforming growth factor $\beta$ on endothelial cells. Proc Natl Acad Sci USA 1987, 84:5600–5604
8. Roberts AB, Sporn MB: Regulation of endothelial cell growth, architecture, and matrix synthesis by TGF-$\beta^1$. Am Rev Respir Dis 1989, 140:1126–1128

9. Pepper MS, Belin D, Montesano R, Orci L, Vassali J-D: Transforming growth factor $\beta$ modulates basic fibroblast growth factor-induced proteolytic and angiogenic properties of endothelial cells in vitro. J Cell Biol 1990, 111:743–755
10. Folkman J, Klagsburn M: Angiogenic factors. Science 1987, 235: 442–447
11. Meininger CJ, Zetter BR: Mast cells and angiogenesis. Semin Cancer Biol 1992, 3:73–79
12. O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M., Lane WS, Cao Y, Sage EH, Folkman J: Angiostatin: a novel angiogenesis inhibitor that mediate the suppression of metastases by Lewis lung carcinoma. Cell 1994, 79:315–328
13. Gately S, Twardowsky P, Stack MS, Patrick M, Boggio L, Cundiff DL, Schnaper HW, Madison L, Volpert O, Bouck N, Enghild J, Kwaan HC, Soff GA: Human prostate carcinoma cells express enzymatic activity that converts human plasminogen to the angiogenesis inhibitor, angiostatin. Cancer Res 1996, 56:4887–4990
14. Good DJ, Polverini PJ, Rastinejad F, LeBeau, MM, Lemons RS, Frazier WA, Bouck NP: A tumor suppressor dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. Proc Natl Acad Sci USA 1990, 87: 6624–6628
15. DiPietro LA, Polverini PJ: Angiogenic macrophages produce the angiogenic inhibitory thrombospondin 1. Am J Pathol 1993, 43:678–684
16. O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR, Folkman J: Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell 1997, 88:277–285
17. Polverini PJ: Macrophage induced angiogenesis: a review. Cytokines 1989, 1:54–73
18. DiPietro LA, Polverini PJ: Role of macrophage in the positive and negative regulation of wound neovascularization. Behring Inst Mitt 1993, 92:238–247
19. Folkman J: The role angiogenesis in tumor growth. Semin Cancer Biol 1992, 3:65–71
20. Iruela-Arispe ML, Dvorak HF: Angiogenesis: a dynamic balance of simulators and inhibitors. Thromb Haemost 1997, 78:672–677
21. Nguyen M, Stubel NA, Bischoff J: A role for sialyl Lewis-X/A glycoconjugates in capillary morphogenesis. Nature 1996, 365:267–269
22. Koch AE, Halloran MM, Haskel CJ, Shah NR, Polverini PJ: Angiogenesis mediated by soluble forms of E-selectin and vascular cell adhesion molecule 1. Nature 1995, 376:517–519
23. Morbidelli L, Brogelli L, Granger HJ, Ziche M: Endothelial cell migration is induced by soluble P-selectin. Life Sci 1998, 62:7–11
24. Nguyen M, Eilber FR, Defrees S: Novel synthetic analogs of sialyl Lewis X can inhibit angiogenesis in vitro and in vivo. Biochem Biophys Res Commun 1996, 228:716–723
25. Banks RE, Gearing AJ, Hemingway IK, Norfolk DR, Perren TJ, Selby PJ: Circulating intercellular adhesion molecule-1 (ICAM-1), E-selectin and vascular cell adhesion molecule-1 (VCAM-1) in human malignancies. Br J Cancer 1993, 68:122–124
26. Wittig BM, Kaulen H, Thees R, Schmitt C, Knolle P, Stock J, Meyer zum Buschenfelde KH, Dippold W: Elevated serum E-selectin in patients with liver metastases of colorectal cancer. Eur J Cancer 1996, 32A:1215–1218
27. Matsuura N, Narita T, Mitsuoka C, Kimura N, Kannagi R, Imai T, Funahashi H, Takagi H: Increased concentrations of soluble E-selectin in the sera of breast cancer patients. Anticancer Res 1997, 17: 1367–1372
28. Hebbar M, Revillion F, Louchez MM, Vilain O, Fournier C, Bonneterre J, Peyrat J-P: The relationship between concentrations of circulating soluble E-selectin and clinical, pathological, and biological features in patients with breast cancer. Clin Cancer Res 1998, 4:373–380
29. Hartwell DW, Butterfield CE, Frenette PS, Kenyon BM, Hynes RO, Folkman J, Wagner DD: Angiogenesis in P- and E-selectin-deficient mice. Microcirculation 1998, 5:173–178
30. Gerritsen ME, Shen CP, Atkinson WJ, Padgett RC, Gimbrone MA Jr, Milstone DS: Microvascular endothelial cells from E-selectin-deficient mice form tubes in vitro. Lab Invest 1996, 75:175–184
31. Nguyen M, Folkman J, Bischoff J: 1-Deoxymannojirimycin inhibits capillary tube formation in vitro analysis of linked oligosaccharides in bovine capillary endothelial cells. J Biol Chem 1992, 267:26157–26165
32. Barondes SH, Castronovo V, Cooper DN, Cummings RD, Drickamer K, Feizi T, Gitt MA, Hirabayashi J, Hughes C, Kasai K-I, Leffler H, Liu FT, Lotan R, Mercurio AM, Monsigny M, Pillai S, Poiere F, Raz A, Rigby PW, Rini JM, Wang JL: Galectins—a family of animal $\beta$-galactoside-binding lectins. Cell 1994, 76:597–598
33. Barondes SH, Cooper DNW, Gitt MA, Leffler H: Galectins, structure, and function of a large family of animal lectins. J Biol Chem 1994, 269:20807–20819
34. Akahani S, Nangia-Makker P, Inohara H, Kim H-RC, Raz A: Galectin-3, a novel anti-apoptotic molecule with a functional BH1 (NWGR) domain of Bcl-2 family. Cancer Res 1997, 57:5272–5276
35. Lotan R, Ito H, Yasui W, Yokozaki H, Lotan D, Tahara E: Expression of a 31 kDa lactose-binding lectin in normal human gastric mucosa and in primary and metastatic gastric carcinomas. Int J Cancer 1994, 56:474–480
36. Shoeppner HL, Raz A, Ho SB, Bresalier R: Expression of an endogenous galactoside-binding lectin correlates with neoplastic progression in the colon. Cancer 1995, 75:2818–2826
37. Xu X-C, El-Nagger A, Lotan R: Differential expression of galectin-1 and galectin-3 in thyroid tumors: potential diagnostic implications. Am J Pathol 1995, 147:815–822
38. Nangia-Makker P, Thompson EW, Hogan C, Ochieng J, Raz A: Induction of tumorigenicity by gal-3 in a non-tumorigenic human breast carcinoma cell line. Int J Oncol 1995, 7:1079–1087
39. Gillenwater A, Xu C, El-Nagger AK, Clayman GN, Lotan R: Expression of galectins in head and neck squamous cell carcinoma. Head Neck 1990, 18:422–432
40. Fernandez PL, Merino MJ, Gomez M, Campo E, Medina T, Castronovo V, Sanjuan X, Liu F-T, Sobel M: Galectin-3 and laminin expression in neoplastic and non-neoplastic thyroid tissue. J Pathol 1997, 181:80–86
41. Bresalier RS, Mazurek N, Sternberg LR, Byrd JC, Yunker CK, Nangia-Makker P, Raz A: Metastasis of human colon cancer is altered by modifying expression of the $\beta$ galactoside-binding protein galectin-3. Gastroenterology 1998, 115:287–296
42. Nangia-Makker P, Sarvis R, Visscher DW, Bailey-Penrod, J, Raz A, Sarkar FH: Galectin-3 and L1 retrotransposons in human breast carcinomas. Breast Cancer Res Treat 1998, 49:171–183
43. Ochieng J, Platt D, Tait L, Hogan V, Raz T, Carmi P, Raz A: Structure function relationship of a recombinant human galactoside-binding protein. Biochemistry 1993, 32:4455–4460
44. Platt D, Raz A: Modulation of the lung colonization of B16–F1 melanoma cells by citrus pectin. J Natl Cancer Inst 1992, 84:438–442
45. Bauer J, Margolism M, Schreiner C, Edgell C-J, Azizkhan J, Lazarowski E, Juliano RL: In vitro model of angiogenesis using a human endothelium derived permanent cell line contributions of induced gene expression, G proteins, and integrins. J Cell Physiol 1992, 153:437–449
46. Scatchard G: The attractions of proteins for small molecules and ions. Ann NY Acad Sci 1949, 51:660–672
47. Passaniti A, Taylor RM, Pili R, Guo Y, Long PV, Haney JA, Pauly RR, Grant DS, Martin GR: A simple quantitative method for assessing angiogenesis and anti-angiogenic agents using reconstituted basement membrane, heparin and fibroblast growth factor. Lab Invest 1992, 67:519–528
48. Inohara I, Raz A: Effects of natural complex carbohydrate (citrus pectin) on murine melanoma cell properties related to galectin-3 functions. Glycoconj J 1994, 11:527–532
49. Pienta KJ, Naik H, Akhtar A, Yamazaki K, Replogle TS, Lehr J, Donat TL, Tait L, Hogan V, Raz A: Inhibition of spontaneous metastasis in a rat prostate cancer model by oral administration of modified citrus pectin. J Natl Cancer Inst 1995, 87:348–353
50. Bussolino F, Mantovani A, Persico G: Molecular mechanisms of blood vessel formation. Trends Biochem Sci 1997, 22:251–256
51. Polverini PJ: The pathophysiology of angiogenesis. Crit Rev Oral Biol Med 1995, 6:230–247
52. Polverini PJ, Leibovich SJ: Induction of neovascularization in vivo and endothelial proliferation in vitro by tumor associated macrophages. Lab Invest 1984, 51:635–642
53. Polverini PJ: How the extracellular matrix and macrophages contribute to angiogenesis-dependent diseases. Eur J Cancer 1996, 32A: 2430–2437
54. Leek RD, Lewis CE, Whitehouse R, Greenall M, Clarke J, Harris AL: Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. Cancer Res 1996, 56:4625–4629
55. Nangia-Makker P, Ochieng J, Christman JK, Raz A: Regulation of the

expression of galactoside-binding lectin during human monocytic differentiation. Cancer Res 1993, 53:5033–5037
56. Liu F-T, Hsu DK, Zuberi RI, Kuwabara I, Chi EY, Henderson WR: Expression and function of galectin-3, a $\beta$-galactoside-binding lectin, in human monocytes and macrophages. Am J Pathol 1995, 147: 1016–1028
57. Carlson A, Collin P, Leffler H, Dahgren C: Galectin-3 activates the NADPH-oxidase in exudated but not peripheral blood neutrophils. Blood 1998, 91:3430–3438
58. Sato S, Hughes RC: Binding of a baby hamster kidney lectin for H type I and II chains, polylactosamine glycans, and appropriately glycosylated forms of laminin and fibronectin. J Biol Chem 1992, 267:6983–6990
59. Warfield PR, Nangia-Makker P, Raz A, Ochieng J: Adhesion of human breast carcinoma to extracellular matrix proteins is regulated by galectin-3. Invasion Metastasis 1997, 17:101–112
60. Kern FG, Lippman ME: The role of angiogenic growth factors in breast cancer progression. Cancer Metastasis Rev 1996, 15:213–219
61. Enenstein J, Waleh NS, Kramer RH: Basic FGF and TGF-$\beta$ differentially modulate integrin expression of human microvascular endothelial cells. Exp Cell Res 1992, 203:499–503
62. Bischoff J: Cell adhesion and angiogenesis. J Clin Invest 1997, 99:373–376
63. Bach TL, Barsigian C, Chalupowics DG, Busler D, Yaen CH, Grant DS, Martinez J: VE-cadherin mediates endothelial cell capillary tube formation in fibrin and collagen gels. Exp Cell Res 1998, 238:324–334
64. Vlodavsky I, Folkman J, Sullivan R, Fridman R, Ishai MR, Sasse J, Klagsbrun M: Endothelial cell derived basic fibroblast growth factor: synthesis and deposition into subendothelial extracellular matrix. Proc Natl Acad Sci USA 1987, 84:2292–2296
65. Kjellen L, Lindahl U: Proteoglycans: structure and interactions. Annu Rev Biochem 1991, 60:443–475
66. Vlodavsky I, Miao H-Q, Medalion B, Danagher P, Ron D: Involvement of heparan sulfate and related molecules in sequestration and growth promoting activity of fibroblast growth factor. Cancer Metastasis Rev 1996, 15:177–186