

Mechanical Entrapment Is Insufficient and Intercellular Adhesion Is Essential for Metastatic Cell Arrest in Distant Organs¹

Olga V. Glinskii^{*,†}, Virginia H. Huxley^{*,‡}, Gennadi V. Glinsky[§], Kenneth J. Pienta[¶], Avraham Raz[#] and Vladislav V. Glinsky^{†,**}

*Department of Medical Pharmacology and Physiology, University of Missouri, Columbia, MO 65212, USA;

†Harry S. Truman Memorial Veterans Hospital, Columbia, MO 65201, USA; ‡Dalton Cardiovascular Research

Center, University of Missouri, Columbia, MO 65212, USA; §Sidney Kimmel Cancer Center, San Diego,

CA 92121, USA; ¶Departments of Internal Medicine and Urology, University of Michigan, Ann Arbor, MI 48109,

USA; #Department of Pathology and Karmanos Cancer Institute, Wayne State University, Detroit, MI 48201, USA;

**Department of Biochemistry, University of Missouri, Columbia, MO 65212, USA

Abstract

In this report, we challenge a common perception that tumor embolism is a size-limited event of mechanical arrest, occurring in the first capillary bed encountered by blood-borne metastatic cells. We tested the hypothesis that mechanical entrapment alone, in the absence of tumor cell adhesion to blood vessel walls, is not sufficient for metastatic cell arrest in target organ microvasculature. The *in vivo* metastatic deposit formation assay was used to assess the number and location of fluorescently labeled tumor cells lodged in selected organs and tissues following intravenous inoculation. We report that a significant fraction of breast and prostate cancer cells escapes arrest in a lung capillary bed and lodges successfully in other organs and tissues. Monoclonal antibodies and carbohydrate-based compounds (anti-Thomsen-Friedenreich antigen antibody, anti-galectin-3 antibody, modified citrus pectin, and lactulosyl-L-leucine), targeting specifically β -galactoside-mediated tumor-endothelial cell adhesive interactions, inhibited by >90% the *in vivo* formation of breast and prostate carcinoma metastatic deposits in mouse lung and bones. Our results indicate that metastatic cell arrest in target organ microvessels is not a consequence of mechanical trapping, but is supported predominantly by intercellular adhesive interactions mediated by cancer-associated Thomsen-Friedenreich glycoantigen and β -galactoside-binding lectin galectin-3. Efficient blocking of β -galactoside-mediated adhesion precludes malignant cell lodging in target organs.

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the mechanical trapping theory and the seed and soil hypothesis, suggested by James Ewing and Stephen Paget, and further enhanced and developed by Isaiah J. Fidler, Lance A. Liotta, Garth Nicolson, and others (reviewed in Refs. [1,2]). Recent advances in intravital video microscopy techniques shed light onto numerous critical steps in tumor metastasis [3–9], suggesting that early metastasis-associated events, including initial micrometastases growth, may occur entirely intravascularly [3–5]. Nevertheless, despite extensive investigative efforts, several important questions related to how tumor cells lodge in a target organ vasculature remain unresolved.

For example, based on video microscopic observations, several groups suggested that metastatic cell arrest is a highly efficient, strictly mechanical process occurring in the first capillary bed encountered due to size limitation [5–7]. Thus, many view mechanical tumor embolism as an ultimate cause of metastatic deposit formation. However, recent results from Vantuyghem et al. [8] documented the development of macroscopic extrapulmonary metastasis in the ovaries, peritoneal cavities, and abdominal mesenteries of mice injected intravenously with B16F10 melanoma cells. Given that survival of early metastatic colonies is a highly inefficient process [6], these results imply that a significant number of injected cells escaped mechanical arrest in the pulmonary microcirculation and landed successfully in other organs and tissues. Therefore, neoplastic cell arrest in the first capillary bed encountered could be a significantly less efficient process

Abbreviations: TF antigen, Thomsen-Friedenreich antigen; FBS, fetal bovine serum; MCP, modified citrus pectin; Lactulosyl-L-leucine; *N*-(1-deoxy-4-*O*-(β -D-galactopyranos-1-yl)-D-fructofuranos-1-yl)-(5)-2-amino-4-methylpentanoic acid

Address all correspondence to: Vladislav V. Glinsky, Department of Biochemistry, University of Missouri, 117 Schweitzer Hall, Columbia, MO 65211. E-mail: glinskiiv@missouri.edu

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Introduction

The current vision of the process of hematogenous cancer metastasis is based largely on two fundamental premises:

than previously thought. Our recent results, showing that metastatic cancer cells are capable of avoiding mechanical entrapment by adjusting their shape and passing through narrow microcirculatory compartments [9], further support this idea.

Taken together, these observations led us to hypothesize that mechanical factors alone are not sufficient for the ultimate tumor cell arrest in target organ circulation, and that specific adhesive interactions between metastatic cells and blood vessel endothelia are necessary for malignant cell arrest in microvessels. Indeed, many metastatic cells arrested in lung circulation, as shown recently by Al-Mehdi et al. [3] and Wong et al. [4], reside in precapillary arterioles of calibers far exceeding tumor cells in size. Similarly, Orr and Wang [10] documented colon carcinoma cell arrest in precapillary hepatic vessels larger than tumor cell diameters. In our experiments employing porcine dura mater model [11], we observed frequently stable breast and prostate cancer cell adhesion within 50- to 100- μm -wide precapillary arterioles [9,11,12]. These facts demonstrate unambiguously that blood-borne malignant cells could be arrested in a variety of organs and tissues through specific adhesive interactions with vessel walls in the absence of mechanical entrapment.

Further, *in vitro* metastatic breast and prostate carcinoma cell adhesion to microvascular endothelium derived from anatomic sites, including the lungs and bone marrow, is mediated largely by interactions between cancer-associated Thomsen-Friedenreich (TF) glycoantigen (Gal β 1-3GalNAc) and β -galactoside-binding lectin galectin-3 [9,11–17]. We also demonstrated that *in vitro* TF antigen/galectin-3 interactions could be disrupted efficiently using function-blocking antibodies against galectin-3 [13,17] and TF antigen [12,15], or small-molecular-weight carbohydrate-based inhibitors specifically targeting β -galactoside-mediated adhesion such as modified citrus pectin (MCP) and lactulosyl-L-leucine (*N*-(1-deoxy-4-*O*-(β -D-galactopyranos-1-yl)-D-fructofuranos-1-yl)-(S)-2-amino-4-methylpentanoic acid) [15–17]. Thus, to test our hypothesis, we investigated *in vivo* patterns of organ-to-organ distribution of fluorescently labeled breast and prostate cancer cells, injected intravenously in mice. In the same model, we determined whether blocking of β -galactoside-mediated adhesion modified the formation of metastatic deposits in target organ microvasculature.

Materials and Methods

Antibodies, Chemicals, and Reagents

TIB-166, H18/7, DREG56, and WAPS 12.2 hybridoma cell lines, producing function-blocking monoclonal antibodies directed against galectin-3, E-selectin, L-selectin, and P-selectin, respectively, were obtained from ATCC (Manassas, VA). The JAA-F11 hybridoma producing anti-TF antigen mAb [18] was kindly provided by Dr. Kate Rittenhouse-Olson (State University of New York, Buffalo, NY). All hybridoma cell lines were grown using exactly the same media composition [RPMI 1640 medium supple-

mented with L-glutamine, 10% fetal bovine serum (FBS), sodium pyruvate, and nonessential amino acids]. Thus, when conditioned supernatants were used in *in vivo* metastatic deposit formation assay, they served as negative controls to each other. MCP and lactulosyl-L-leucine were obtained as described previously [19–22]. All other chemicals and reagents, unless otherwise specified, were from Sigma (St. Louis, MO).

Cancer Cell Lines and Cultures

The MDA-MB-435 human breast carcinoma cell line was kindly provided by Dr. Janet E. Price (M. D. Anderson Cancer Center, Houston, TX). The DU-145 human prostate carcinoma cells were purchased from ATCC. The RPMI 1640 medium supplemented with L-glutamine, 10% FBS, sodium pyruvate, and nonessential amino acids was used for tumor cell lines.

In Vivo Metastatic Deposit Formation Assay

Six-week-old male (for prostate cancer experiments) or female (for breast cancer experiments) HsdJcr:Ha(ICR)-scid mice (Harlan, Indianapolis, IN) were used in this study, in accordance with the University of Missouri-approved animal care protocol. Prior to intravenous injection, cancer cells were pre-labeled for 5 minutes with 3 $\mu\text{g}/\text{ml}$ solution of acridine orange in RPMI 1640 medium, rinsed three times with serum-free RPMI 1640 medium, and dissociated from plastic using a nonenzymatic cell dissociation reagent (Sigma). We demonstrated previously that labeling tumor cells with acridine orange does not affect their adhesive behavior in short time experiments [12]. Immediately following the dissociation, tumor cells were resuspended using one of the following: 1) complete RPMI 1640 medium (control); 2) complete RPMI 1640 medium supplemented with MCP (0.25% wt/vol final concentration); 3) complete RPMI 1640 medium supplemented with lactulosyl-L-leucine (2 mM final concentration); or 4) undiluted conditioned supernatant containing a corresponding function-blocking antibody directed against galectin-3, TF antigen, or E-selectin, L-selectin, or P-selectin, and pipetted to produce a single cell suspension. All subsequent manipulations with cancer cells were performed using the same media composition. To remove any remaining cell clumps, the tumor cell suspension was filtered through a 20- μm nylon mesh, and adjusted to contain 5×10^6 cells/ml. Next, 200 μl (1×10^6 cells) of a single-cell suspension of fluorescently labeled MDA-MB-435 or DU-145 cells was injected into a lateral tail vein of the experimental animal. Three hours postinjection, the animals were euthanized, and internal organs [lung, bones (vertebrae and sternum), liver, spleen, kidney, thyroid gland, and brain] were removed, examined by epifluorescence microscopy, and photographed using a QICAM high-performance digital CCD camera (Quantitative Imaging Corporation, Burnaby, Canada). At least two identical experiments were performed for each experimental set-up. In each animal, subpleural metastatic deposits were scored in four random observation fields. The results were calculated and presented as mean \pm SD.

Results and Discussion

Metastatic Cell Arrest in the First Capillary Bed Encountered Is Less Efficient than Previously Thought

To test the hypothesis that a significant number of circulating neoplastic cells are capable of escaping mechanical entrapment in the first capillary bed encountered, we investigated *in vivo* patterns of organ-to-organ distribution of intravenously injected fluorescently labeled breast (MDA-MB-435) and prostate (DU-145) cancer cells. Three hours postinjection, numerous metastatic cells landed in the lungs (Figure 1A). However, as predicted, a significant number of tumor cells avoided arrest in lung circulation and lodged in bones (Figure 1, B and C). In addition, selected neoplastic cells were found in other organs such as the kidneys and thyroid gland (Figure 1, D and E). Of note, our experiments yielded comparable metastatic deposit counts in the lungs and bones (vertebrae), which did not differ significantly from each other (Figure 1F). These results demonstrate that neoplastic cell arrest in the first capillary bed encountered (lung) is less efficient than previously thought, suggesting that mechanical entrapment due to size limitation is not the ultimate cause of tumor cell arrest in lung.

Thus, we have reasoned that specific adhesive interactions between metastatic cells and microvascular endothelia, rather than mechanical factors, could play the foremost role in mediating tumor cell arrest in distant organ microvessels. Recent results from our groups revealed that, *in vitro*, metastatic breast and prostate carcinoma cell adhe-

sion to microvascular endothelial cells derived from various anatomic sites, including the lungs [17] and bone marrow [13,15], mediated largely by interactions between cancer-associated TF glycoantigen and β -galactoside-binding lectin galectin-3 [13–17]. Therefore, we investigated next whether blocking TF antigen and galectin-3 with monoclonal antibodies would modify the formation of breast and prostate cancer metastatic deposits in the lungs and bones *in vivo*.

Anti-TF Antigen and Anti-Galectin-3 Antibodies Inhibit Lung and Bone Metastatic Deposit Formation

In vitro, TF antigen/galectin-3 interactions mediate heterotypic adhesion between tumor cells and endothelia [9,15–17], as well as malignant cell homotypic aggregation with each other [9,15–17,23], both of which may contribute to metastatic cell arrest in microcirculation [9]. Further, galectin-3 expressed on both neoplastic and endothelial cells participates in these processes [9,17]. Thus, we investigated next whether blocking TF antigen and galectin-3 with monoclonal antibodies modified the formation of breast and prostate cancer metastatic deposits in the lungs and bones *in vivo*. In these experiments, conditioned supernatants of hybridoma cultures were used as function-blocking antibodies. All five hybridoma cell lines used in this study were grown using exactly the same media composition (RPMI 1640 medium supplemented with L-glutamine, 10% FBS, sodium pyruvate, and nonessential amino acids). The ability of TIB-166 and JAA-F11 antibodies, directed against galectin-3 and TF antigen, respectively, to inhibit tumor cell adhesion to the endothelium *in vitro* was demonstrated previously [12,15,17]. The supernatants of hybridomas producing antibodies directed against E-selectin, L-selectin, and P-selectin were tested for their ability to inhibit the rolling of peripheral mononuclear cells on TNF- α - or histamine-activated endothelial monolayers and P-selectin-coated plates, respectively. Consequently, all antibodies were applied at the concentrations exceeding their related *in vitro* IC₅₀ at least 50- to 100-fold. Thus, when conditioned supernatants were used in *in vivo* metastatic deposit formation assay (Figures 2 and 3), they served as negative controls to each other.

The results of these experiments demonstrated that blocking either TF antigen or galectin-3 with monoclonal antibodies dramatically inhibits metastatic deposit formation in both the lung and bones (Figure 2). It appears that efficient blockage of TF antigen/galectin-3-mediated adhesion precludes almost completely metastatic breast and prostate cancer cell arrest in distant organ microvessels. This outcome indicates that such arrest is consequent to adhesive interactions between tumor cells and microvascular endothelia, and not mechanical entrapment.

Intravenous injection of the fluorescently labeled tumor cells yields consistently reproducible counts of subpleural metastatic deposits in mouse lungs, and allows for assessing the inhibitory effect of various agents on this process. Thus, in the next series of experiments, we focused specifically on quantifying the effect of several potential antiadhesion compounds on subpleural metastatic deposit formation.

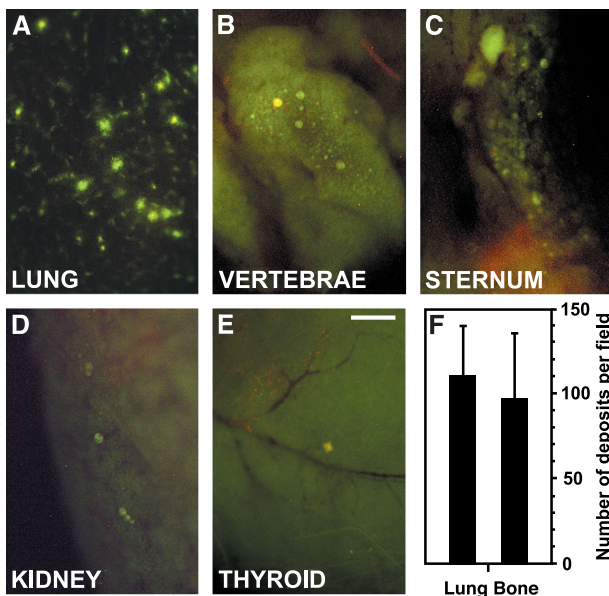


Figure 1. A significant fraction of intravenously injected cancer cells escapes mechanical entrapment in the first capillary bed encountered (lung) and reaches other organs and tissues. Metastatic deposits of fluorescently labeled cancer cells (DU-145 human prostate carcinoma shown) formed *in vivo* 3 hours postinjection of 1×10^6 cells in the lungs (A), vertebrae (B), sternum (C), kidney (D), and thyroid gland (E). Scale bar, 500 μ m. (F) Metastatic deposit counts in lungs and bones (vertebrae). Bars, mean \pm SD.

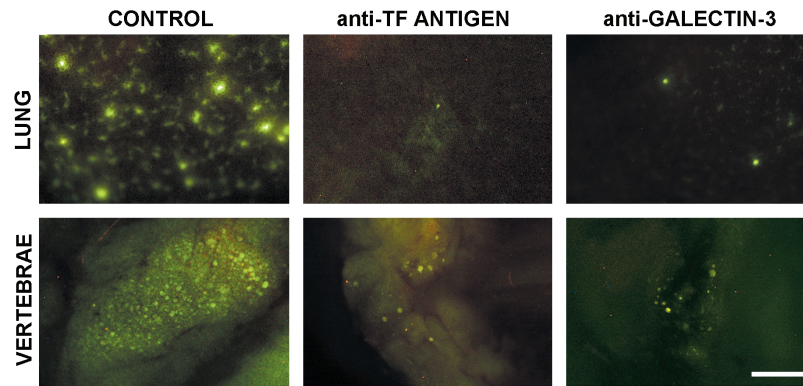


Figure 2. The effect of anti-TF antigen and anti-galectin-3 antibodies on metastatic deposit formation in the lungs and bones *in vivo*. Both anti-TF antigen and anti-galectin-3 function-blocking monoclonal antibodies dramatically inhibit metastatic deposit formation in mouse lungs (top panel) and bones (bottom panel) *in vivo*. Scale bar, 500 μ m.

Breast and Prostate Cancer Cell Arrest in Murine Lung Supported Predominantly by β -Galactoside-Mediated Interactions

In these experiments, in addition to anti-TF antigen and anti-galectin-3 antibodies, two small-molecular-weight carbohydrate-based compounds, lactulosyl-L-leucine and MCP, were tested along with the function-blocking antibodies directed against E-selectin, L-selectin, and P-selectin for their ability to inhibit the formation of subpleural metastatic deposits. Of these, lactulosyl-L-leucine and MCP bind galectins and inhibit β -galactoside-mediated adhesion by mimicking essential structural features of naturally occurring carbohydrate structures [15,16]. For example, lactulosyl-L-leucine specifically blocks galectin-3 by mimicking cancer-associated TF antigen [15]. Importantly, these carbohydrate-based galectin-3 inhibitors were already shown to reduce efficiently both the incidence and number of spontaneous breast and prostate carcinoma metastases *in vivo* [16,20,22].

The results of these experiments demonstrated that all four compounds targeting β -galactoside-mediated interactions (anti-TF antigen, anti-galectin-3, lactulosyl-L-leucine, and MCP) inhibited almost completely the formation of breast and prostate carcinoma subpleural metastatic deposits *in vivo* (Figure 3). Specifically, anti-TF mAb, anti-galectin-3 mAb, lactulosyl-L-leucine, and MCP inhibited subpleural metastatic deposit formation by $99.3 \pm 1.11\%$, $97.5 \pm 2.26\%$, $96.3 \pm 3.21\%$, and $92.7 \pm 4.88\%$ (mean \pm SD), and $99.8 \pm 0.32\%$, $97.6 \pm 0.96\%$, $94.8 \pm 6.65\%$, and $91.7 \pm 7.30\%$ (mean \pm SD) of breast and prostate carcinoma cells, respectively. Thus, MDA-MB-435 and DU-145 metastatic cell arrest in lung microvessels is mediated predominantly by cancer-associated TF glycoantigen and galectin-3. Furthermore, although the inhibitory effect of these compounds on bone colonization was not quantified, our results (Figure 2, *d-f*) strongly suggest that β -galactoside-mediated intercellular adhesion is likely to play a pivotal role in supporting the homing of breast and prostate carcinoma cells to the bone microvasculature as well. However, in addition to inhibiting cell-to-cell adhesion, blocking galectin-3 may potentially increase tumor cell susceptibility to apoptosis including

anoikis and, therefore, affect their viability and impact experimental outcomes. To ensure that the inhibitors of TF antigen/galectin-3 interactions do not affect cancer cell viability, we performed a series of control experiments, in which single-cell suspensions of tumor cells were incubated for 3 hours at 37°C in 5% CO₂ atmosphere without (control) or with anti-TF antigen, anti-galectin-3, lactulosyl-L-leucine, and MCP at the same concentrations as in *in vivo* experiments. To prevent cancer cell adhesion to the plastic, these experiments were conducted in ultralow adhesion plates. The percentage of viable cells determined by a Trypan blue dye exclusion after 3 hours of incubation was as follows: control, $94.7 \pm 4.8\%$; JAA-F11 (anti-TF antigen), $92.3 \pm 5.2\%$; TIB-166 (anti-galectin-3), $90.9 \pm 8.1\%$; lactulosyl-L-leucine, $92.3 \pm 7.9\%$; and MCP, $88.7 \pm 7.5\%$ (mean \pm SD). These results indicate that the galectin-3 and TF antigen inhibitors used in this study do not significantly affect tumor cell viability and, therefore, their effects on metastatic deposit formation in this experimental system could be attributed entirely to their antiadhesion effect. In contrast, it appears that selectins do not play a major role in breast and prostate carcinoma cell

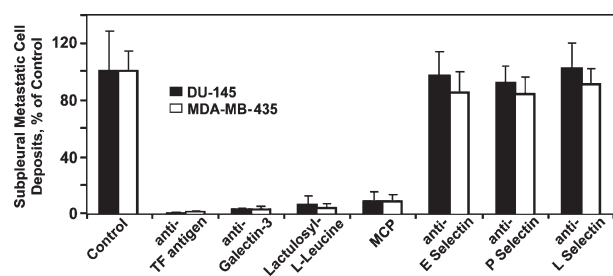


Figure 3. The effect of various function-blocking monoclonal antibodies and carbohydrate-based compounds on subpleural metastatic deposit formation of DU-145 human prostate carcinoma and MDA-MB-435 human breast carcinoma cells. Anti-TF antigen, anti-galectin-3, lactulosyl-L-leucine, and MCP inhibit >90% subpleural metastatic deposit formation of prostate (closed bars) and breast (open bars) cancer cells *in vivo*, whereas all three of antiselectin antibodies tested fail to affect this process significantly. Bars, mean \pm SD.

arrest in murine lung microvasculature. All three antiselectin antibodies tested failed to affect this process significantly (Figure 3). These results are consistent with observations by Satoh et al. [24] that multiple prostate carcinoma cell lines lack selectin-mediated adhesion despite expression of a sialyl-Lewis(x) antigen (the ligand for endothelial selectins). However, selectin-mediated adhesion plays an important role in colon cancer cell arrest in hepatic microvessels (Ref. [10] and reviewed in Ref. [25]). Similarly, in different types of cancer, other adhesion molecules could be crucial for metastatic cell lodging in their related target tissues.

The notion that mechanical entrapment is the primary means of metastatic cell arrest in distant organ microvessels is based largely on the fact that blood-borne metastatic cells lodge predominantly in precapillary vessels and capillaries (reviewed in Refs. [1,2]). In the absence of efficient inhibitors of tumor–endothelial cell adhesion, this phenomenon was interpreted by many as an evidence of tumor cell mechanical arrest in target organ microvessels. Here, we demonstrate that efficient blocking of tumor–endothelial cell adhesion precludes almost completely metastatic cell arrest and retention in lung and bones. It appears that mechanical tumor embolism does not occur when intercellular adhesive interactions are blocked, indicating that mechanical entrapment is not sufficient for the ultimate tumor cell arrest in microcirculation. Thus, we suggest that mechanical factors play a rather supportive role in mediating metastatic cell lodging in distant organs (i.e., mechanical factors reduce tumor cell traveling velocities and prolong neoplastic cell contact with microvascular endothelium, increasing greatly the chances for adhesive interactions to take place). However, as suggested previously by Fidler and Talmadge [26] in murine melanoma model, mechanical factors may cause an arrest of multicellular aggregates. We recently reported that both breast and prostate cancer cells form such aggregates intravascularly at the sites of their primary attachment to the endothelium [9] and, lately, the formation of multicellular metastatic deposits *in vivo* by human fibrosarcoma cells was elegantly shown by Yamamoto et al. [27] using color-coded fluorescently labeled cancer cells.

Here, we propose the model whereby mechanical and adhesive factors act cooperatively to support metastatic cell arrest in target organ microvessels. On the molecular level, we believe that TF antigen/galectin-3 interactions represent some of the earliest events in a multistep cell-to-cell adhesion process. Most likely, these carbohydrate–lectin interactions, which are rather weak and transient in nature, are important in initiating tumor–endothelial cell adhesive cascade and in mediating subsequent integrin-mediated stabilizing steps, which further determine the fate of metastatic deposits and organ specificity of hematogenous cancer metastasis. For example, the results from Wang et al. [28] demonstrated an important role for $\alpha_3\beta_1$ integrin in mediating the pulmonary arrest of several cancer cell lines, including the MDA-MB-435 breast carcinoma cells, which were used in this study. Further, most recently, Fukushi et al. [29] showed that galectin-3 physically associates with $\alpha_3\beta_1$ integrin at the endothelial cell membrane. Taken together, these facts

suggest a logical chain of subsequent molecular events, whereas TF antigen expressed on tumor cells mediates galectin-3 clustering on endothelial cell surfaces [9,12,15] and initiates transient adhesion, which is stabilized by $\alpha_3\beta_1$ integrin engagement and association with galectin-3. The galectin-3–mediated involvement of $\alpha_3\beta_1$ integrin provides for both the means of anchoring galectin-3 at the cell membrane and for initiating multiple downstream signaling cascades. In our opinion, this model offers answers to important questions regarding the microvascular pathophysiology of hematogenous cancer spread, and provides further justification for the unification of the seed and soil and mechanical trapping theories of cancer metastasis.

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