

# The Role of Thomsen-Friedenreich Antigen in Adhesion of Human Breast and Prostate Cancer Cells to the Endothelium<sup>1</sup>

Vladislav V. Glinsky, Gennadi V. Glinsky, Kate Rittenhouse-Olson, Margaret E. Huflejt, Olga V. Glinskii, Susan L. Deutscher, and Thomas P. Quinn<sup>2</sup>

Department of Biochemistry, University of Missouri, Columbia, Missouri 65211 [V. V. G., O. V. G., S. L. D., T. P. Q.]; Cancer Research Center, Columbia, Missouri 65201 [V. V. G.]; Sidney Kimmel Cancer Center, San Diego, California 92121 [G. V. G., M. E. H.]; Metastat, Inc., San Diego, California 92121 [G. V. G.]; and Department of Biotechnical and Clinical Laboratory Sciences, State University of New York at Buffalo, Buffalo, New York 14214 [K. R.-O.]

## ABSTRACT

Interactions of metastatic cancer cells with vasculature endothelium are critical during early stages of cancer metastasis. Understanding the molecular underpinnings of these interactions is essential for the development of new efficacious cancer therapies. Here we demonstrate that cancer-associated carbohydrate T antigen plays a leading role in docking breast and prostate cancer cells onto endothelium by specifically interacting with endothelium-expressed  $\beta$ -galactoside-binding protein, galectin-3. Importantly, T antigen-bearing glycoproteins are also capable of mobilizing galectin-3 to the surface of endothelial cells, thus priming them for harboring metastatic cancer cells. The T antigen-mediated, tumor-endothelial cell interactions could be efficiently disrupted using synthetic compounds either mimicking or masking this carbohydrate structure. High efficiency of T antigen-mimicking and T antigen-masking inhibitors of tumor cell adhesion warrants their further development into antiadhesive cancer therapeutics.

## INTRODUCTION

Metastasis is a major fatal complication associated with human malignant disorders. Recent findings demonstrate that hematogeneous cancer metastases originate from intravascular growth of endothelium-attached rather than extravasated cancer cells (1), highlighting the key role of tumor-endothelial cell interactions in cancer metastasis. Understanding the molecular mechanisms of these interactions is crucial for the development of new efficacious cancer therapies. A broad array of adhesion molecules, such as carbohydrates, lectins, cadherins, and integrins, have been implicated in the adhesion of tumor cells to the vasculature endothelium. This reflects a complexity of the adhesion process. Moreover, different adhesion molecules have been shown to participate at distinct stages in a multistep binding process (2). For example, selectins were shown to contribute to the initial contact of circulating cells with endothelium by inducing their rolling (3), whereas galectin-3 has been proposed to participate in docking of cancer cells on capillary endothelium (4), and integrins were demonstrated to play a role in the development of more stable attachment involving protein-protein interactions (3).

Among all of the variety of adhesion molecules, cell surface carbohydrate structures have been a focus of many investigative efforts into their roles in cancer cell adhesion and metastasis. Aberrations in cell surface carbohydrates, emblematic of malignant transformation, have been shown to facilitate tumor cell colonization and metastasis by effecting normal cell-cell interactions (5, 6). One of the most widely distributed cancer-associated cell surface carbohydrate moi-

eties is the pancarcinoma T antigen<sup>3</sup> (7). T antigen is a simple mucin-type disaccharide (8), Gal $\beta$ 1–3GalNAc, expressed on the outer cell surfaces of T-cell lymphomas and most human carcinomas (7–9), including breast and prostate (10). The role for T antigen in tumor cell adhesion and metastasis has been proposed based on the existence of T antigen-mediated adhesion of highly metastatic murine lymphoma cells and hepatocytes (9). Recently, we demonstrated the participation of T antigen in human breast carcinoma cell adhesion to the endothelium (11). We also reported that a 15-amino acid T antigen-specific peptide, P-30 (HGRFILPWWYAFSPS), selected from a bacteriophage display library (12, 13), specifically and significantly (>50%) inhibited adhesion of human breast cancer cells to the endothelium (11). These results underscored the importance of T antigen-mediated interactions in breast cancer metastasis. However, the molecular mechanisms of T antigen-mediated adhesion as well as cognate physiological receptors for T antigen have not been identified.

On the basis of the fact that the terminal residue of T antigen is  $\beta$ -galactose, we investigated its potential interactions with galectin-3 (11), a  $M_r \sim 30,000$  member of a family of soluble  $\beta$ -galactoside-specific lectins (14, 15). Although the physiological role of this carbohydrate-binding protein is still greatly debated, galectin-3 has been implicated in several distinct fundamental cellular processes such as pre-mRNA splicing (16), cell growth and differentiation (17), regulation of apoptosis (18, 19), and cell-cell recognition and adhesion (19–21). In several experimental systems, the expression of galectin-3 in cancer cells was associated with increased malignant and metastatic phenotype (22, 23). Moreover, preferential adhesion of PC-3 human prostate cancer cells to bone marrow endothelial cells was found to be at least in part galectin-3-dependent (4). Recently, we demonstrated the involvement of the endothelium-expressed galectin-3 in breast carcinoma-endothelial cell adhesion (11) supportive of a possible interaction of this  $\beta$ -galactoside-binding lectin with T antigen.

We hypothesized that if T antigen is indeed interacting with galectin-3 during tumor cell docking onto the endothelium, it could be a common molecular mechanism of cancer cell adhesion pertinent to a metastatic dissemination of a variety of T antigen-expressing human malignancies. Carbohydrate-lectin interactions are believed to take place during an initial reversible phase of cell adhesion that determines cell-cell recognition specificity (24, 25). The efficient specific blockage of these early binding events may significantly modify the outcome of the adhesion and affect the metastatic process in whole.

In this study, we demonstrate that two synthetic inhibitors of T antigen-mediated adhesion, the T antigen-binding P-30 peptide, and a sugar-based T antigen mimetic, lactulosyl-L-leucine, efficiently (43–56%) inhibit the adhesion of breast and prostate carcinoma cells to

Received 11/28/00; accepted 4/18/01.

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

<sup>1</sup> Supported in part by United States Army Grant DAMD17-97-1-7198 and Department of Energy Grant ER-61661 (to T. P. Q.), Department of Defense Grant DAMD17-98-1-8320, and a grant from the Department of Veterans Affairs (to S. L. D.). V. V. G. was supported in part by the Cancer Research Center.

<sup>2</sup> To whom requests for reprints should be addressed, at Department of Biochemistry, University of Missouri, 117 Schweitzer Hall, Columbia, MO 65211. Phone: (573) 882-6099; Fax: (573) 884-4812; E-mail: QuinnT@missouri.edu.

<sup>3</sup> The abbreviations used are: T antigen, Thomsen-Friedenreich antigen; HUVEC, human umbilical vein endothelial cell; HBMEC, human bone marrow endothelial cell; FBS, fetal bovine serum; PNA, peanut agglutinin; DAB, diamino benzidine tetrahydrochloride; DiI, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine; ASF, asialofetuin; T-PAA, T antigen-polyacrylamide; T-HSA, T antigen-human serum albumin; lactulosyl-L-leucine, *N*-(1-deoxy-4-O-( $\beta$ -D-galactopyranosyl-1-yl)-D-fructofuranosyl-1-yl)-(S)-2-amino-4-methylpentanoic acid.

HUVEC monolayer. Both compounds exhibit an even more pronounced effect on cancer cell adhesion to human bone marrow microvascular endothelium (74–82% and 69–70% inhibition for breast and prostate carcinoma cells, respectively). These results strongly suggest possible involvement of T antigen-mediated adhesive interactions in breast and prostate cancer metastatic bone disease. Both T antigen-masking P-30 and T antigen-mimicking lactulosyl-L-leucine display the same maximal inhibitory effect on adhesion of breast and prostate cancer cells as a highly specific anti-T antigen monoclonal antibody does. The results of inhibition ELISA experiments confirm the specificity of both synthetic inhibitors and demonstrate direct binding of the recombinant human galectin-3 to the protein-linked T antigen. Remarkably, endothelial cells exhibit rapid and marked increase in cell surface galectin-3 expression when treated with T antigen-bearing glycoproteins. This observation suggests a novel function for circulating T antigen-expressing glycoproteins such as cancer-associated mucin, which may act by priming capillary endothelium for harboring cancer cells.

The results presented in this paper define T antigen as one of the leading factors during early stages of breast and prostate cancer-endothelial cell interactions. We demonstrate that T antigen is acting both as a major cell surface carbohydrate ligand for galectin-3 on breast and prostate cancer cells and as a factor causing mobilization of galectin-3 to the outer membrane on endothelial cells. The significance of T antigen-mediated adhesion in breast and prostate cancer identifies T antigen-galectin-3 interactions as a valid target for development of new antiadhesive therapies of cancer metastases.

## MATERIALS AND METHODS

**Antibodies, Chemicals, and Reagents.** The monoclonal anti-T antigen antibody was developed as described (26). The TIB-166 hybridoma, producing rat monoclonal anti-galectin-3 (anti-Mac-2) was purchased from American Type Culture Collection. Goat Texas Red-conjugated antirat antibody was purchased from Molecular Probes (Eugene, OR). The monoclonal antibody to irrelevant plant protein was kindly provided by the Cell and Immunobiology Core (University of Missouri, Columbia, MO). The *Escherichia coli* strains, expressing human galectin-3 from pET5 vector, were kindly provided by Dr. A. Raz (Wayne State University, Detroit, MI) and Dr. H. Leffler (Lund University, Lund, Sweden). Recombinant human galectin-3 was isolated and affinity purified as described (27). DiI was from Molecular Probes (Eugene, OR). Biotinylated T-PAA was from Glycotech Corp. (Rockville, MD). T-HSA was from Dextra Laboratories (Reading, United Kingdom). T antigen binding peptide P-30 (HGRFILPWYAFSPS) and control peptide (RRLLFYKYVYKRYRAGKQRG) were chemically synthesized using FMOC-based chemistry and purified to homogeneity on a C-18 reverse-phase HPLC column (ISCO Corp.). All other chemicals and reagents, unless otherwise specified, were purchased from Sigma Chemical Co. (St. Louis, MO).

**Cell Lines and Cultures.** The MDA-MB-435 human breast carcinoma cell line was kindly provided by Dr. J. Price (M. D. Anderson Cancer Center, Houston, TX). The DU-145 human prostate carcinoma cells were obtained from American Type Culture Collection (Rockville, MD). B16-F10, a highly metastatic variant of B16-F1 mouse melanoma, was kindly provided by Dr. I. Fidler (M. D. Anderson Cancer Center). The RPMI 1640 supplemented with L-glutamine, 10% FBS, sodium pyruvate, and nonessential amino acids, was used for all tumor cell lines.

HUVECs were purchased from Cascade Biologicals (Portland, OR). Basal Medium 200 (Cascade Biologicals) supplemented with low serum growth supplement containing FBS (final concentration, 2% v/v), hydrocortisone, human fibroblast growth factor, heparin, and human epidermal growth factor was used for HUVECs. The cells at population doublings of approximately 8–12 were used for the adhesion experiments.

HBMEC line HBMEC-60 was kindly provided by Dr. C. E. van der Schoot (University of Amsterdam, Amsterdam, the Netherlands). This cell line was developed by immortalization of HBMECs originally isolated from adult human bone marrow using the amphotrophic helper-free retrovirus pLXSN16

E6/E7 (28). The HBMEC-60 cells were shown to maintain their normal phenotype and adhesive properties, specifically the ability to bind hematopoietic progenitor cells (28). Basal Medium 200 (Cascade Biologicals) supplemented with 20% FBS and low serum growth supplement containing hydrocortisone, human fibroblast growth factor, heparin, and human epidermal growth factor was used for HBMEC-60. All cells were maintained on plastic as a monolayer culture in a humidified incubator in 5% CO<sub>2</sub>/95% air at 37°C.

**Adhesion to the Endothelium.** The adhesion experiments were performed exactly as described (11). A single cell suspension of cancer cells prelabeled with 3 μg/ml solution of acridine orange or 5 μg/ml solution of DiI in RPMI 1640 (5 × 10<sup>4</sup> cells/chamber in 2.5 ml of complete media supplemented with various concentrations of the compound tested) was added to the monolayer of the endothelial cells grown to confluence directly on microscope slides using 4-well chamber slides (NalgeNunc, Naperville, IL). The chambers were sealed, and cells were allowed to adhere for 1 h at 37°C, after which the chambers were inverted and left upside-down for 30 min to allow sedimentation of nonadhered cells. After that, the medium was drained, samples were gently rinsed with PBS, fixed for 30 min in 2% formaldehyde solution in PBS, mounted under coverglass, and examined by fluorescence microscopy. Four random fields in each well were photographed at ×250, and the total number of adhered cells in every field was counted. The assay was performed in quadruplicate for each condition.

**T Antigen Cell Binding Assay.** The B16-F10 mouse melanoma cell line was selected for these experiments because it does not express T antigen (12), which could interfere with the assay, but is capable of binding T antigen, as reflected by its interaction with ASF (13). The cells were placed on a microscope slide, fixed for 30 min in 2% formaldehyde solution in PBS, and incubated for 1 h in PBS containing biotinylated T-PAA (final concentration, 20 μg/ml) with or without lactulosyl-L-leucine (final concentration, 2 mM) at room temperature. Cells were washed three times with PBS. Bound T-PAA was visualized using peroxidase-labeled streptavidin and DAB as a chromogenic substrate. After counterstaining with hematoxylin, samples were mounted under coverglass and analyzed microscopically at ×900.

**Dot Blot Assay.** Bovine ASF, bearing multiple T antigen epitopes, was immobilized on the nitrocellulose membrane (1 μg/spot). After blocking with 2% solution of BSA in TBS, samples were incubated for 1 h at room temperature with different concentrations of peroxidase-labeled T antigen-specific PNA lectin without (control) or with addition of 2 mM (final concentration) of lactulosyl-L-leucine or lactitol-L-leucine in 2% BSA solution in TBS. The membranes were washed three times for 10 min each time in TBS and developed with DAB.

**Inhibition ELISA.** T-HSA (250 ng/well) was preabsorbed to 96-well plates overnight, blocked with 1% solution of BSA in PBS, and incubated for 1 h with 10 μg/ml solution of purified galectin-3 (1 μg/well) in the presence of different concentrations of T antigen-specific P-30 peptide (0–50 μM) or lactulosyl-L-leucine (0–1 mM) at room temperature. Bound galectin-3 was detected by sequential incubation with rat anti-galectin-3 monoclonal antibody, alkaline phosphatase conjugated goat anti-rat IgG, and *p*-nitrophenyl phosphate, followed by reading the absorbance at 405 nm. The plates were washed three times with PBS between steps. Controls containing no galectin-3 were included as blanks. Data represent the results of at least three determinations for each condition. The inhibition percentage was calculated as a decrease in the mean net absorbance at 405 nm compared with control.

**Cytochemistry, Laser Scanning Confocal Microscopy, and Immunofluorescence.** The cytochemical analysis of T antigen was performed using PNA-horseradish peroxidase conjugate and subsequent color reaction with DAB.

The samples for the analysis of galectin-3 cellular distribution were prepared exactly as described in adhesion to the endothelium, except that cancer cells were not prelabeled, and samples were fixed and permeabilized in 4% formaldehyde solution in PBS for 24 h. The rat anti-galectin-3 and goat Texas Red-conjugated antirat antibodies were used to visualize galectin-3. The laser scanning confocal microscopy was performed on a Bio-Rad MRC 600 confocal system. The Z stacks were prepared by obtaining serial sections with 0.5 μm increments and analyzed in orthogonal projections (Y-Z and X-Z sections) using the MetaMorph Imaging System software (Universal Imaging, Hallis, NH).

To evaluate cell surface galectin-3 expression, live, nonfixed, and nonpermeabilized endothelial cells, grown to confluence directly on microscope slides, were incubated for 45 min at 37°C in complete medium supplemented

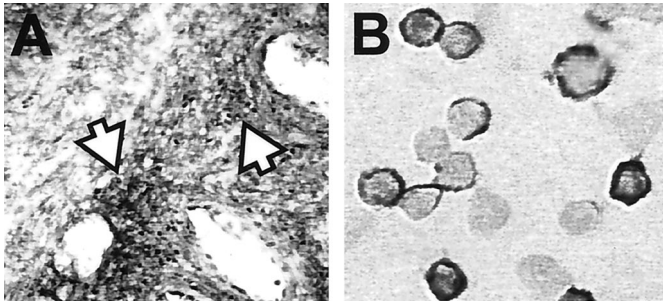


Fig. 1. Expression of T antigen in a human prostate cancer lesion (A) and in the DU-145 human prostate adenocarcinoma cell line (B) as revealed by binding of T antigen-specific PNA lectin-horseradish peroxidase conjugate followed by color reaction with diaminobenzidine tetrahydrochloride. Arrows, areas of a positive staining corresponding to the brown color, indicating the presence of immunoreactive T antigen.

with rat anti-galectin-3 antibody without (control) or with 1 mg/ml (final concentration) of bovine fetuin or ASF, or 0.5 mg/ml (final concentration) of HSA or T-HSA. Slides were washed three times with PBS and incubated for 1 h with goat Texas Red-conjugated antirat antibody. After additional washes, the samples were mounted under coverglass and analyzed on a Bio-Rad MRC 600 confocal system using accumulation of four scans. The four-well slides (NalgeNunc) were used, which allowed simultaneous processing of samples to be compared. The exact same conditions, such as iris settings, laser intensity, gain, magnification, and number of scan accumulations, were used for all samples.

## RESULTS AND DISCUSSION

**T Antigen Expression.** The expression of T antigen in breast cancer tissues is well documented and is associated with tumor progression and metastasis (29). However, this carbohydrate structure is not specific only to breast cancer. Rather, it is characteristic of a vast majority of human adenocarcinomas. Similarly, T antigen is often detectable in prostate cancer lesions (Fig. 1A). Furthermore, in patients with prostate carcinoma, the expression of T antigen has also been found to correlate with tumor grade and metastasis (10, 30). Both breast (MDA-MB-435) and prostate (DU-145) cancer cell lines used in this study express T antigen on their surfaces. We demonstrated previously the presence of T antigen on MDA-MB-435 human breast carcinoma cells (13). This cell line, originally isolated from the

pleural effusion of a patient with breast cancer, has been shown to be highly metastatic in nude mice (31, 32). The fact that adhesion of MDA-MB-435 cells to the endothelium could be efficiently (2-fold) inhibited by the T antigen-binding P-30 peptide (11) is indicative of the importance of T antigen in breast cancer-endothelial cell interactions. The DU-145 human prostate carcinoma cell line, chosen for our experiments, was also originally isolated from a metastatic lesion (33). Importantly, this cell line retains its metastatic potential, as reflected in the ability of DU-145 cells to develop metastasis in nude mice (34). In this study, we show that similar to MDA-MB-435 metastatic breast cancer cells, the DU-145 human prostate carcinoma cells also express T antigen on their surfaces (Fig. 1B). This observation suggests that T antigen might likewise participate in prostate cancer cell adhesion to the endothelium. To address this question, we investigated whether compounds, capable of specifically masking T antigen, would interfere with prostate cancer-endothelial cell interactions.

**Inhibition of Cancer Cell Adhesion to HUVECs by Masking T Antigen.** The T antigen-specific P-30 peptide (HGRFILPWWYAF-SPS) was originally isolated from a bacteriophage display library (12). The synthetic P-30 binds with high affinity and specificity to both free T antigen disaccharide in solution and T antigen-bearing glycoproteins (13). It is also capable of specifically recognizing T antigen-expressing cancer cells of different origin (12, 13) and efficiently inhibiting ASF-mediated cancer cell aggregation (13) and breast carcinoma cell adhesion to the endothelium (11). Our previous results suggest that the peptide is masking T antigen epitopes on cancer cells, thus preventing interactions with their cognate ligands. In this study, we used the T antigen-masking P-30 peptide to investigate whether T antigen participates in prostate cancer cell adhesion to the endothelium. We studied the effect of different concentrations (0–0.1 mg/ml) of synthetic T antigen-specific P-30 on adhesion of DU-145 human prostate carcinoma cells to a monolayer of HUVECs. The results of these experiments (Fig. 2, A–H) showed that P-30 inhibited the adhesion of both DiI-labeled (Fig. 2A) and acridine orange-labeled (Fig. 2, B–G) DU-145 cells to endothelial cells in a dose-dependent manner, whereas the control peptide (RLLLFYKYVYKRYRAG-KQRG) failed to inhibit adhesion (Fig. 2H). To assess the efficiency of T antigen masking by P-30 peptide, we compared the maximal inhibitory effects on breast and prostate cancer cell binding to the

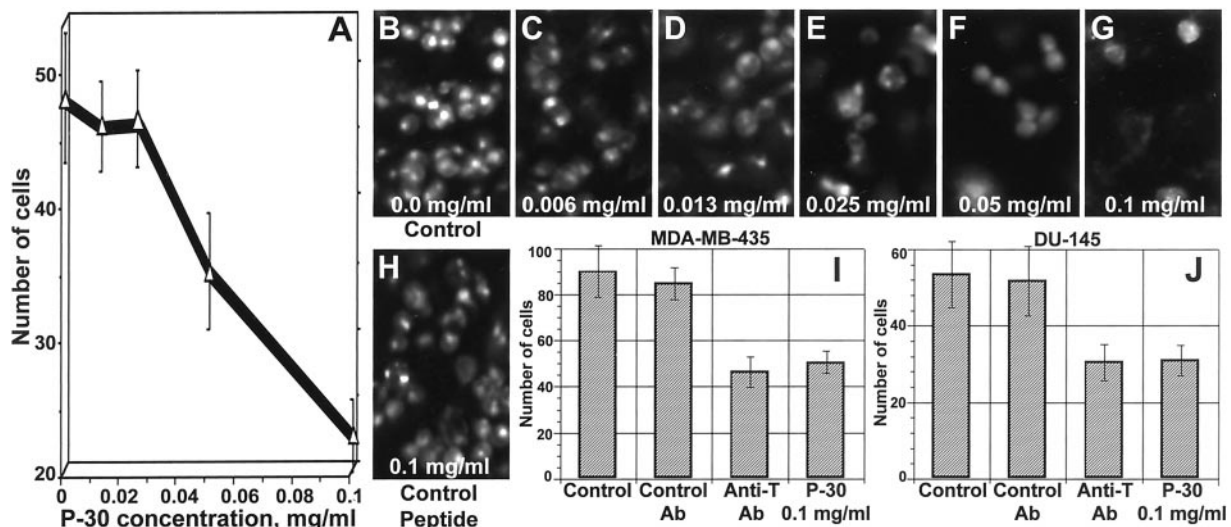


Fig. 2. Dose-dependent inhibition of DiI-labeled (A) and acridine orange-labeled (B–G) DU-145 human prostate carcinoma cell adhesion to the endothelium by synthetic T antigen-specific P-30 peptide but not by the control peptide (H). In B–H, numbers at the bottom indicate the concentration of the peptide tested. I and J, maximal inhibitory effect on adhesion of MDA-MB-435 human breast carcinoma (I) and DU-145 human prostate carcinoma (J) cells to the endothelium achievable with anti-T antigen monoclonal antibody and P-30 peptide; bars, SD.



endothelium, achievable with P-30 and an anti-T antigen antibody. A monoclonal antibody to irrelevant plant protein was used as a control in these experiments. We found that the control antibody did not inhibit attachment of tumor cells to HUVECs (Fig. 2, *I* and *J*), whereas both anti-T antigen antibody and P-30 exhibited almost identical maximal inhibitory effect on breast (Fig. 2*I*) and prostate (Fig. 2*J*) cancer cell adhesion. The results of these experiments demonstrated that synthetic P-30 peptide was masking T antigen as efficiently as the highly specific monoclonal anti-T antigen antibody. However, neither P-30 nor anti-T antigen antibody inhibited tumor cell adhesion to the endothelium completely. The inhibition efficiency (ranging from 43 to 56% in different experiments) displayed by these two compounds most likely reflected the impact of T antigen-mediated interactions in our experimental system. Apparently, different adhesion molecules such as integrins might also contribute to the binding. It has been suggested that circulating metastatic cells interact with endothelium in two distinct stages (24): the initial reversible docking stage, mediated by carbohydrate-lectin interactions; and the second stabilizing integrin-mediated locking stage, which requires more prolonged cell contact (25). In our adhesion experiments, cancer and endothelial cells were in physical contact for 1 h, allowing sufficient time for integrin-mediated binding events to occur. Supportive of this suggestion are results reported by Lehr and Pienta (4) using a similar experimental design. They demonstrated that antibodies to different members of the integrin family, such as CD11a, CD18, and leukocyte function antigen-1, inhibited adhesion of PC-3 prostate cancer cells to endothelium from 20 to 55%, suggesting the importance of integrins in stabilizing adhesion of tumor cells docked onto the endothelium. In contrast, interactions between T antigen and the corresponding carbohydrate-binding lectin(s) preceding integrin-mediated adhesion most likely are crucial during an initial docking stage. Supportive of this idea are the results of experiments using in-flow experimental systems,<sup>4</sup> showing that under conditions of flow, anti-integrin antibodies do not affect the adhesion of MDA-MB-435 breast carcinoma cells to the endothelium, whereas the synthetic T antigen mimetic lactulosyl-L-leucine inhibits it up to 80%. These results imply that T antigen, but not integrin-mediated interactions, plays a significant role in initiating cancer cell binding to the endothelium.

**Inhibition of Cancer Cell Adhesion to HUVECs by Mimicking T Antigen.** An independent approach to the study of T antigen-mediated adhesion would be to use compounds capable of mimicking T antigen. One such compound is a synthetic carbohydrate-amino acid conjugate (glycoamine), lactulosyl-L-leucine. Lactulosyl-L-leucine is capable of targeting  $\beta$ -galactoside-binding lectins (galectins) by mimicking the corresponding carbohydrate epitopes of naturally occurring glycoproteins (35). Similar to P-30, lactulosyl-L-leucine efficiently inhibits ASF-mediated cancer cell aggregation and competes with T antigen-specific PNA lectin for binding to breast carcinoma cells (36). In this study, we report that lactulosyl-L-leucine likewise inhibited the adhesion of breast (Fig. 3, *E* and *F*) and prostate (data not shown) tumor cells to the endothelium. The inhibition efficiency of this compound, 52 and 54% for breast and prostate carcinoma cells, respectively, was similar to that of the P-30 peptide and the anti-T antigen antibody. However, unlike P-30, lactulosyl-L-leucine is acting not through masking but through mimicking T antigen. The T antigen mimetic properties of lactulosyl-L-leucine are evident in its ability to compete off binding of T antigen to cancer cells (Fig. 3, *A–C*) and T

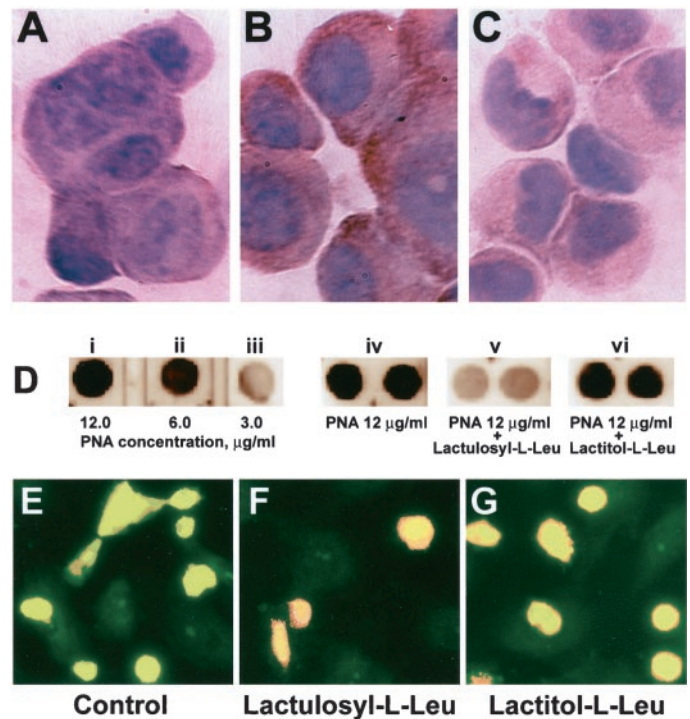


Fig. 3. T antigen-mimicking lactulosyl-L-leucine competes off biotinylated T antigen-PAA conjugate (*C*) bound to the B16-F10 mouse melanoma cells (*B*). *A*, control sample counterstained with hematoxylin. *D*, inhibition of T antigen-specific PNA lectin binding to ASF (*iv*) by T antigen-mimicking lactulosyl-L-leucine (*v*) but not by lactitol-L-leucine (*vi*); *i–iii*, binding of different concentrations of PNA lectin to ASF (1  $\mu$ g) spotted on the nitrocellulose membrane. *E–G*, inhibition of DiI-labeled MDA-MB-435 breast carcinoma cell adhesion to the endothelium by T antigen-mimicking lactulosyl-L-leucine (*F*) but not by lactitol-L-leucine (*G*), compared with the control (*E*).

antigen-specific PNA lectin to ASF (Fig. 3*D*, *iv* and *v*). Interestingly, the substitution of lactulosyl for lactitol completely abolishes the ability of the resulting compound, lactitol-L-leucine, to both mimic T antigen (Fig. 3*D*, *vi*) and inhibit tumor cell adhesion to the endothelium (Fig. 3*G*). These results demonstrate that the antiadhesive properties of lactulosyl-L-leucine strictly depend on the carbohydrate moiety and aptitude of the compound to mimic T antigen. Importantly, this T antigen-mimicking compound was already shown to inhibit up to 75% both the incidence and number of spontaneous breast cancer lung metastases in nude mice experiments (36). This finding highlights the critical role of T antigen-mediated interactions in the metastatic process. In patients, however, both breast and prostate tumors most often metastasize to bone (37). Therefore, it will be very important to investigate whether T antigen is relevant to breast and prostate cancer metastatic bone disease.

**Inhibition of Breast and Prostate Cancer Cell Adhesion to Human Bone Marrow Microvascular Endothelium.** The results of postmortem examination demonstrate that ~70% of patients who die from carcinomas of the breast and prostate have evidence of bone metastases (37). Lately, it was suggested that specific tumor cell adhesive interactions with bone marrow endothelium could be an important factor in the predilection of prostate cancer metastases to the skeleton (4, 38). This hypothesis is strongly supported by the results presented recently by Lehr and Pienta (4). They demonstrated that prostate cancer cells adhere better to the bone marrow-derived endothelium than to the endothelium from other anatomical sites.

To further delineate the role of T antigen in breast and prostate cancer metastasis, we investigated whether T antigen participates in adhesion of MDA-MB-435 breast carcinoma and DU-145 prostate carcinoma cells to the monolayer of HBMEC-60 human bone marrow

<sup>4</sup> S. K. Khaldoyanidi, V. V. Glinsky, L. Sikora, A. B. Glinskii, V. V. Mossine, G. V. Glinsky, and P. Srirammarao. Role of galectin-3 in homo- and heterotypic intercellular adhesion of human metastatic breast carcinoma cells to endothelia under flow conditions, submitted for publication.

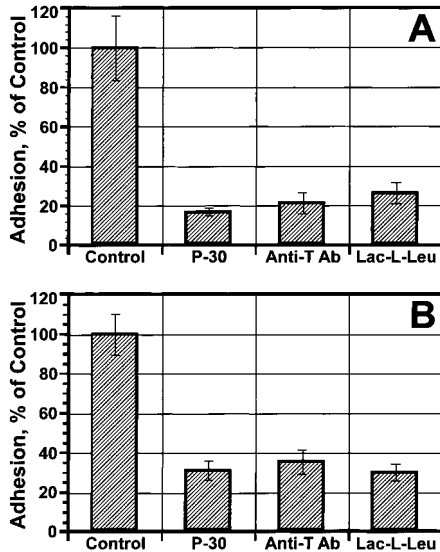


Fig. 4. Inhibition of MDA-MB-435 human breast carcinoma (A) and DU-145 human prostate carcinoma (B) cell adhesion to the monolayer of HBMEC-60 human bone marrow endothelial cells. The adhesion experiments were performed as described in "Materials and Methods" without (Control) or with 50  $\mu\text{M}$  (final concentration) of T antigen-specific P-30 peptide, 1:2 dilution of anti-T antigen monoclonal antibody, or 1 mM (final concentration) of T antigen-mimicking lactulosyl-L-leucine. The adhesion was calculated as a percentage of control. The results of one of two identical experiments are shown; bars, SD.

endothelial cells. The results of these experiments (Fig. 4) show that both T antigen-masking (P-30 peptide and anti-T antigen antibody) and T antigen-mimicking (lactulosyl-L-leucine) compounds inhibit cancer cell adhesion to bone marrow endothelium even greater than to HUVECs (74–82% and 69–70% inhibition for breast and prostate carcinoma cells, respectively). These data indicate that T antigen-mediated interactions may play an important role in tumor cell adhesion to bone marrow microvasculature; however, the molecular basis of these interactions is still poorly understood. The identification of the molecules acting as T antigen receptors in cancer-endothelial cell adhesion will be of utmost interest.

**Interaction of T Antigen with Galectin-3.** Because the terminal sugar of T antigen is  $\beta$ -galactose, the involvement of  $\beta$ -galactoside-binding lectins (galectins) in T antigen-mediated adhesion was investigated. Two of nine currently known galectins, *i.e.*, galectin-1 and galectin-3, were found previously to be most prominently expressed in cancer and endothelial cells and were implicated in cancer cell adhesion (39–41). On the basis of the fact that human galectin-3 exhibits 200-fold higher specific activity toward Gal $\beta$ 1–3GalNAc disaccharide than galectin-1 (14), we proposed previously the interaction of T antigen with galectin-3 during cancer cell adhesion to the endothelium (11). Supportive of this interaction is the observation reported by Bresalier *et al.* (42) that colon cancer mucin, a glycoprotein most often decorated with multiple T antigen epitopes, serves as a specific ligand for galectin-3. Moreover, they also demonstrated that fully glycosylated mucin binds >40-fold more galectin-3 than mucin from the cells in which oligosaccharide synthesis is blocked at the stage of addition of  $\beta$ 3-linked galactose to the *N*-acetylgalactosamine (Tn antigen) to form T antigen. We reasoned that if T antigen is in fact interacting with galectin-3, then both T antigen-masking and T antigen-mimicking compounds should specifically inhibit this interaction. To test this hypothesis, we performed inhibition ELISA experiments in which T antigen conjugated to HSA was preabsorbed on plastic in 96-well plates and incubated with purified recombinant human galectin-3 in the presence of different concentrations of the P-30 peptide or lactulosyl-L-leucine. We found that both compounds inhibited gal-

tin-3 binding to T antigen-HSA conjugate in a dose-dependent manner (Fig. 5). Although the effect of both compounds was saturable, neither one reached 100% inhibition. We attribute the remaining binding (~30%) to cooperative interactions characteristic of galectin-3 (17). To the best of our knowledge, this is the first observation that directly demonstrated galectin-3 binding to protein-linked T antigen. These data also confirm the specificity of both inhibitors tested, T antigen-masking P-30 peptide and T antigen-mimicking lactulosyl-L-leucine.

The role of galectin-3 in prostate cancer cell adhesion was challenged recently, however, by the results reported by Ellerhorst *et al.* (2). They demonstrated that galectin-3 is not present on the cell surface in any of the four prostate cancer lines they tested, including DU-145, which was used in our experiments, and PC-3, which was shown by another group to bind to the endothelium in a galectin-3-dependent manner (4). To clarify this issue, we investigated cellular distribution of the galectin-3 during cancer-endothelial cell interaction.

**Cellular Distribution of Galectin-3 during Cancer Cell Adhesion to the Endothelium.** Laser scanning confocal microscopy was used to study the localization of the galectin-3 in both cancer and endothelial cells upon their interaction. This technique allowed us to analyze the orthogonal plans (*X-Z* and *Y-Z* sections), which included both cancer and endothelial cells, as well as the sites of their contact. The results of these experiments (Fig. 6, A and B) revealed remarkably similar patterns of galectin-3 distribution during DU-145 prostate carcinoma (Fig. 6A) and MDA-MB-435 breast carcinoma (Fig. 6B) cell adhesion to HUVECs. In both cancer cell lines, galectin-3, although expressed at higher levels than in endothelial cells, remained predominantly intracellular. This result is in agreement with observations of Ellerhorst *et al.* (2) and suggests that galectin-3 of cancer cells does not actively participate in their adhesion to the endothelium. In contrast, on endothelial cells we observed the mobilization and clustering of this carbohydrate-binding protein toward the cancer-endothelial cell contacts. This phenomenon is consistent with the interaction of endothelium-expressed galectin-3 with T antigen displayed on the surface of cancer cells and is indicative of its active role in cancer-endothelial cell adhesion. These results also provide a feasible

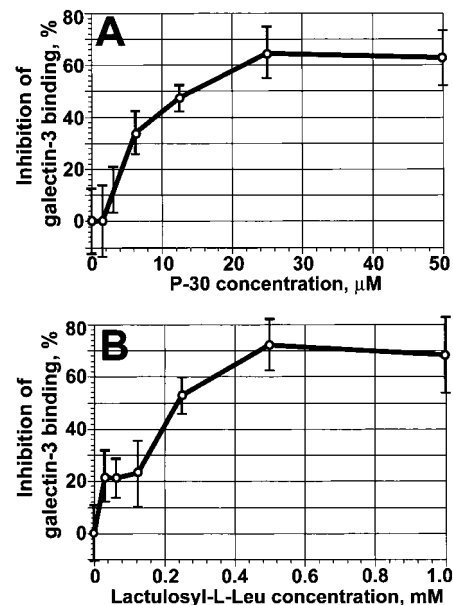


Fig. 5. Dose-dependent inhibition of recombinant human galectin-3 binding to T antigen-HSA by T antigen-masking P-30 peptide and T antigen-mimicking lactulosyl-L-leucine. The inhibition percentage was calculated as a decrease in the mean net absorbance at 405 nm compared with the control; bars, SD.



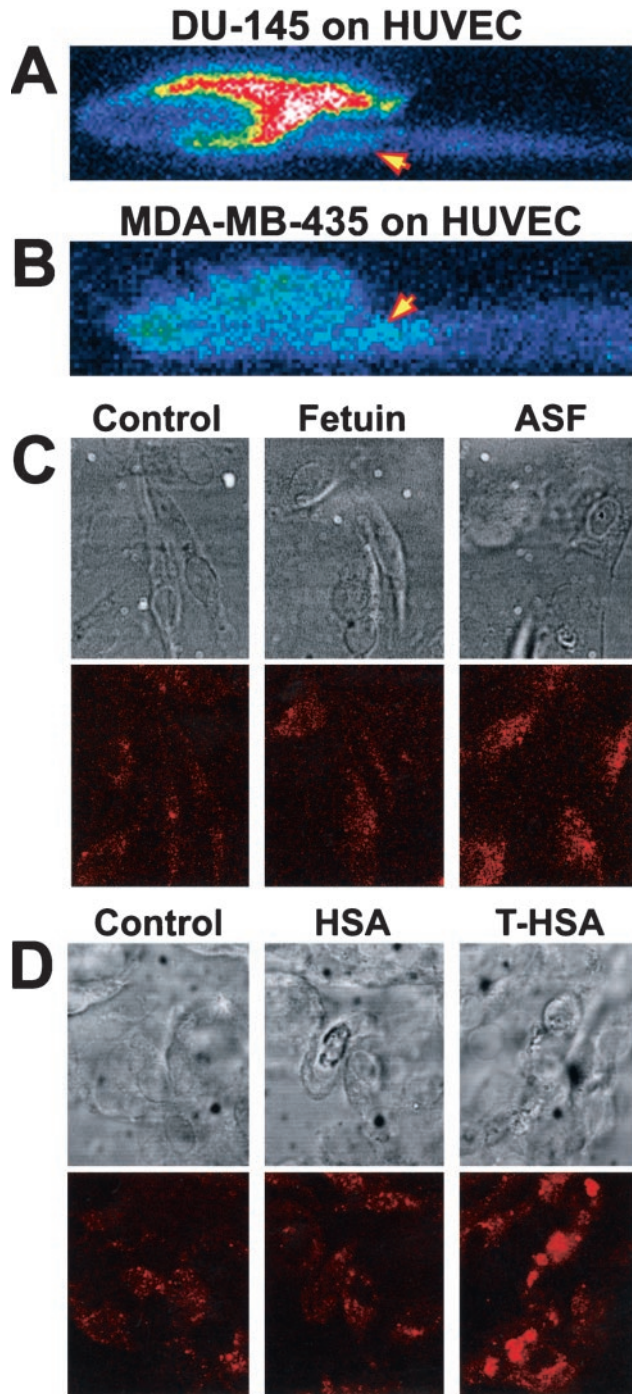


Fig. 6. *A* and *B*, pseudocolored X-Z sections showing cellular distribution of galectin-3 upon adhesion of DU-145 prostate (*A*) and MDA-MB-435 breast (*B*) cancer cells to the endothelium. Different colors represent different concentrations of galectin-3 (purple, blue, green, yellow, red, and white, from lowest to highest). Note the accumulation and clustering of galectin-3 toward cell contacts on endothelial cells indicated by arrows, whereas in cancer cells the protein remains predominantly intracellular. *C* and *D*, the effect of ASF and T-HSA on galectin-3 surface expression on endothelial cells. Note the increase in galectin-3 surface representation upon treatment with ASF (*C*) or T-HSA (*D*) but not with fetuin or HSA, compared with control. In both *C* and *D*, the top panel shows a corresponding transmitted light photomicrograph of the same field.

explanation of why PC-3 cells, which do not exhibit galectin-3 on a cell surface (2), interact with endothelium in a galectin-3-dependent manner, as reported by Lehr and Pienta (4).

The fact that endothelium-expressed galectin-3 clustered toward the sites of cancer-endothelial cell contacts obviously attracted our attention. Thus, we decided to investigate whether T antigen was a factor

causing galectin-3 mobilization and clustering. We studied the changes in galectin-3 surface expression on endothelial cells after treatment with T antigen-bearing glycoproteins, ASF and T-HSA. Both ASF and T-HSA display multiple immunoreactive T antigen epitopes. Two other proteins, bovine fetuin and HSA were used as corresponding negative controls in these experiments. Bovine fetuin is identical to ASF and also contains multiple T antigen antennas, which are, however, covalently masked with sialic (neuraminic) acid and nonimmunoreactive. HSA does not contain T antigen in any form. These experiments revealed that treatment with either ASF (Fig. 6C) or T-HSA (Fig. 6D) resulted in a rapid and significant increase in galectin-3 expression on a surface of endothelial cells, whereas neither fetuin nor HSA effected this expression. These results demonstrate that T antigen is acting not only as a ligand for galectin-3 but also as a factor causing the mobilization of this carbohydrate-binding protein to the cell surface. The latter observation in turn suggests an important novel function for the circulating cancer mucin, which is often found in the serum of patients with adenocarcinomas of different origin (42, 43). Circulating cancer mucin, similar to ASF and T-HSA, bears multiple T antigen moieties and may likewise induce the increase in galectin-3 surface expression on endothelial cells, thus priming vasculature endothelium for binding the circulating metastatic tumor cells. Importantly, this phenomenon could be observed when highly metastatic T antigen-expressing MDA-MB-435 cells interact with microvascular endothelium under conditions of flow.<sup>4</sup> In contrast, the MDA-MB-468 cells, which are deficient in T antigen expression and are nonmetastatic, do not induce galectin-3 surface mobilization. This observation implies that T antigen-expressing circulating metastatic cancer cells could themselves change endothelium adhesiveness.

The fact that T antigen can actually modify the adhesive properties of the endothelium by mobilizing cell surface adhesion molecules such as galectin-3 adds new and important insights into our understanding of tumor-endothelial cell interactions. It suggests the existence of an additional priming stage in adhesion of circulating metastatic cells to the endothelium, which precedes the docking (initial reversible adhesion) and locking (permanent irreversible adhesion) stages (Fig. 7).

The results presented in this report identify T antigen as a major cancer cell surface carbohydrate ligand for galectin-3. Although both the former and the latter may also interact with other cognate ligands, the T antigen-galectin-3 interactions are likely to play a leading role in the initial stages of cancer cell adhesion to the endothelium. The efficient intervention with cancer cell endothelium interactions could result in the development of new antiadhesive cancer therapies (reviewed in Ref. 44), the concepts of which were brought about by early pioneering works of R. Kerbel *et al.* (45, 46) and Inohara and Raz

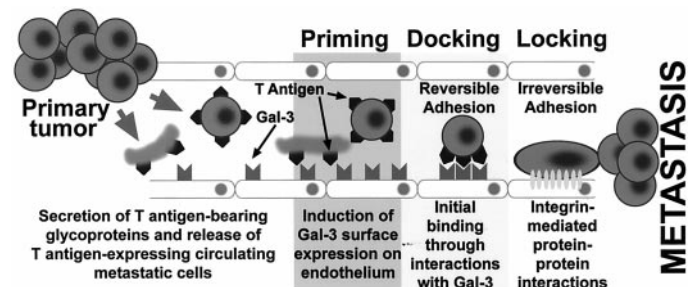


Fig. 7. Schematic representation of T antigen participation in adhesion of metastatic cancer cells to the vasculature endothelium. Circulating T antigen-bearing cancer-associated glycoproteins and metastatic tumor cells induce the mobilization of the galectin-3 to the surface of endothelial cells (*Priming* stage). T antigen-expressing malignant cells bind to the endothelium through T antigen-galectin-3 interactions (*Docking*) allowing sufficient time for the stabilizing integrin-mediated adhesion events to take place (*Locking* stage).

(47). The significant inhibition of breast and prostate cancer cell adhesion to the endothelium by T antigen-masking and T antigen-mimicking compounds suggests their high potential to be developed into efficacious antimetastatic agents.

## ACKNOWLEDGMENTS

We thank Drs. A. Raz and Dr. H. Lefler for the gifts of galectin-3-expressing vectors, Dr. J. E. Price for providing MDA-MB-435 cells, Dr. C. E. van der Schoot for providing HBMEC-60 cells, Dr. I. Fidler for providing B16-F10 cells, and Dr. V. V. Mossine for providing synthetic glycoamines. We also thank the University of Missouri-Columbia Molecular Cytology Core for assistance with confocal microscopy and Cell and Immunobiology Core for help in antibody production.

## REFERENCES

- Al-Mehdi, A. B., Tozawa, K., Fisher, A. B., Shientag, L., Lee, A., and Muschel, R. J. Intravascular origin of metastasis from the proliferation of endothelium-attached tumor cells: a new model for metastasis. *Nat. Med.*, *6*: 100–102, 2000.
- Ellerhorst, J., Nguyen, T., Cooper, D. N. W., Lotan, D., and Lotan, R. Differential expression of endogenous galectin-1 and galectin-3 in human prostate cancer cell lines and effects of overexpressing galectin-1 on cell phenotype. *Int. J. Oncol.*, *14*: 217–224, 1999.
- Lawrence, M. B., and Springer, T. A. Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. *Cell*, *65*: 859–873, 1991.
- Lehr, J. E., and Pienta, K. J. Preferential adhesion of prostate cancer cells to a human bone marrow endothelial cell line. *J. Natl. Cancer Inst.*, *90*: 118–123, 1998.
- Hakomori, S.-I. Aberrant glycosylation in tumors and tumor-associated carbohydrate antigens. *Adv. Cancer Res.*, *52*: 257–331, 1989.
- Hakomori, S.-I. Possible functions of tumor associated carbohydrate antigens. *Curr. Opin. Immunol.*, *48*: 665–673, 1983.
- Springer, G. F., Desai, P. R., Ghazizadeh, M., and Tegtmeier, H. T/Tn pancreatic carcinoma autoantigens: fundamental, diagnostic, and prognostic aspects. *Cancer Detect. Prev.*, *19*: 173–182, 1995.
- Fonseca, I., Costa Rosa, J., Felix, A., Therkildsen, M. H., and Soares, J. Simple mucin-type carbohydrate antigens (T, Tn, and sialosyl-Tn) in mucoepidermoid carcinoma of the salivary glands. *Histopathology (Oxf.)*, *25*: 537–543, 1994.
- Springer, G. F., Cheingsong-Popov, R., Schirmacher, V., Desai, P. R., and Tegtmeier, H. Proposed molecular basis of murine tumor cell-hepatocyte interaction. *J. Biol. Chem.*, *258*: 5702–5706, 1983.
- Janssen, T., Petein, M., Van Velthoven, R., Van Leer, P., Fourmarier, M., Vanegas, J. P., Danguy, A., Schulman, C., Pasteels, J. L., and Kiss, R. Differential histochemical peanut agglutinin stain in benign and malignant human prostate tumors: relationship with prostatic-specific antigen immunostain and nuclear DNA content. *Hum. Pathol.*, *12*: 1341–1347, 1996.
- Glinsky, V. V., Huflejt, M. E., Glinsky, G. V., Deutscher, S. L., and Quinn, T. P. Effects of Thomsen-Friedenreich antigen-specific peptide P-30 on  $\beta$ -galactoside-mediated homotypic aggregation and adhesion to the endothelium of MDA-MB-435 human breast carcinoma cells. *Cancer Res.*, *60*: 2584–2588, 2000.
- Peletskaya, E. N., Glinsky, G. V., Deutscher, S. L., and Quinn, T. P. Identification of peptide sequences that bind the Thomsen-Friedenreich cancer-associated glycoantigen from bacteriophage peptide display libraries. *Mol. Divers.*, *2*: 13–18, 1996.
- Peletskaya, E. N., Glinsky, V. V., Glinsky, G. V., Deutscher, S. L., and Quinn, T. P. Characterization of peptides that bind the tumor-associated Thomsen-Friedenreich antigen selected from bacteriophage display libraries. *J. Mol. Biol.*, *270*: 374–384, 1997.
- Sparrow, C. P., Leffler, H., and Barondes, S. H. Multiple soluble  $\beta$ -galactoside-binding lectins from human lung. *J. Biol. Chem.*, *262*: 7383–7390, 1987.
- Barondes, S. H., Castranovo, V., Cooper, D. N., Cummings, R. D., Drickamer, K., Feizi, T., Gitt, M. A., Hirabayashi, J., Hughes, C., Kasai, K., Leffler, H., Liu, F. T., Lotan, R., Mercurio, M. N., Monsigny, M., Pillai, S., Poirer, F., Raz, A., Rigby, P. W. J., Rini, J. M., and Wang, J. L. Galectins: a family of animal  $\beta$ -galactoside-binding lectins. *Cell*, *76*: 597–598, 1994.
- Dagher, S. F., Wang, J. L., and Patterson, R. J. Identification of galectin-3 as a factor in pre-mRNA splicing. *Proc. Natl. Acad. Sci. USA*, *92*: 1213–1217, 1995.
- Barondes, S. H., Cooper, D. N. W., Gitt, M. A., and Leffler, H. Galectins, structure and function of a large family of animal lectins. *J. Biol. Chem.*, *269*: 20807–20819, 1994.
- Akahani, S., Nangia-Makker, P., Inohara, H., Kim, H.-R. C., and Raz, A. Galectin-3: a novel antiapoptotic molecule with a functional BH1 (NWGR) domain of Bcl-2 family. *Cancer Res.*, *57*: 5272–5276, 1997.
- Kim, H.-R. C., Lin, H.-M., Biliran, H., and Raz, A. Cell cycle arrest and inhibition of anoikis by galectin-3 in human breast epithelial cells. *Cancer Res.*, *59*: 4148–4154, 1999.
- Inohara, H., and Raz, A. Functional evidence that cell surface galectin-3 mediates homotypic cell adhesion. *Cancer Res.*, *55*: 3267–3271, 1995.
- Inohara, H., Akahani, S., Koths, K., and Raz, A. Interactions between galectin-3 and Mac-2-binding protein mediate cell-cell adhesion. *Cancer Res.*, *56*: 4530–4534, 1996.
- Nangia-Makker, P., Sarvis, R., Visscher, D. W., Baily-Penrod, J., Raz, A., and Sarkar, F. H. Galectin-3 and L1 retrotransposons in human breast carcinomas. *Cancer Res. Treat.*, *49*: 171–183, 1998.
- Nangia-Makker, P., Thompson, E., Hogan, C., Ochieng, J., and Raz, A. Induction of tumorigenicity by galectin-3 in a nontumorigenic human breast carcinoma cell line. *Int. J. Oncol.*, *7*: 1079–1087, 1995.
- Honn, K. V., and Tang, D. G. Adhesion molecules and tumor cell interaction with endothelium and subendothelial matrix. *Cancer Metastasis Rev.*, *11*: 353–375, 1992.
- McClay, D. R., Wessel, G. M., and Marchase, R. B. Intercellular recognition: quantitation of initial binding events. *Proc. Natl. Acad. Sci. USA*, *78*: 4975–4979, 1981.
- Rittenhouse-Diakun, K., Xia, Z., Pickhardt, D., Morey, S., Baek, M.-G., and Roy, R. Development and characterization of monoclonal antibody to T-antigen: (Gal $\beta$ 1-3GalNAc- $\alpha$ -O). *Hybridoma*, *17*: 165–173, 1998.
- Ochieng, J., Platt, D., Tait, L., Hogan, V., Raz, T., Carmi, P., and Raz, A. Structure-function relationship of a recombinant human galactoside-binding protein. *Biochemistry*, *32*: 4455–4460, 1993.
- Rood, P. M. L., Calafat, J., Von dem Borne, A. E. G. Kr., Gerritsen, W. R., and van der Schoot, C. E. immortalization of human bone marrow endothelial cells: characterization of new cell lines. *Eur. J. Clin. Investig.*, *30*: 618–629, 2000.
- Wolf, M. F., Ludwig, A., Fritz, P., and Schumacher, K. Increased expression of Thomsen-Friedenreich antigens during tumor progression in breast cancer patients. *Tumor Biol.*, *9*: 190–194, 1988.
- Ghazizadeh, M., Kagawa, S., Izumi, K., and Kurokawa, K. Immunohistochemical localization of T antigen-like substance in benign hyperplasia and adenocarcinoma of the prostate. *J. Urol.*, *132*: 1127–1130, 1984.
- Zhang, R. D., Fidler, I. J., and Price, J. E. Relative malignant potential of human breast carcinoma cell lines established from pleural effusion and a brain metastasis. *Invasion Metastasis*, *11*: 204–215, 1991.
- Price, J. E., Polyzos, A., Zhang, R. D., and Daniels, L. M. Tumorigenicity and metastasis of human breast carcinoma cell lines in nude mice. *Cancer Res.*, *50*: 717–721, 1990.
- Stone, K. R., Mickey, D. D., Wunderli, H., Mickey, G. H., and Paulson, D. F. Isolation of human prostate carcinoma cell line (DU-145). *Int. J. Cancer*, *21*: 274–281, 1978.
- Bladou, F., Gleave, M. E., Penault-Llorca, F., Serment, G., Lange, P. H., and Vessella, R. L. *In vitro* and *in vivo* models developed from human prostatic cancer. *Prog. Urol.*, *7*: 384–396, 1997.
- Glinsky, G. V., Mossine, V. V., Price, J. E., Bielenberg, D., Glinsky, V. V., Ananthaswamy, H. N., and Feather, M. S. Inhibition of colony formation in agarose of metastatic breast carcinoma and melanoma cells by synthetic glycoamine analogs. *Clin. Exp. Metastasis*, *14*: 253–267, 1996.
- Glinsky, G. V., Price, J. E., Glinsky, V. V., Mossine, V. V., Kiriakova, G., and Metcalf, J. B. Inhibition of human breast cancer metastasis in nude mice by synthetic glycoamines. *Cancer Res.*, *56*: 5319–5324, 1996.
- Rubens, R. D. Bone metastases—the clinical problem. *Eur. J. Cancer*, *34*: 210–213, 1998.
- Cooper, C. R., and Pienta, K. J. Cell adhesion and chemotaxis in prostate cancer metastasis to bone: a mini-review. *Prostate Cancer Prostatic Dis.*, *3*: 6–12, 2000.
- Raz, A., and Lotan, R. Endogenous galactoside-binding lectins: a new class of functional tumor cell surface molecules related to metastasis. *Cancer Metastasis Rev.*, *6*: 433–452, 1987.
- Lotan, R., and Raz, A. Endogenous lectins as mediators of tumor cell adhesion. *J. Cell. Biochem.*, *37*: 107–117, 1988.
- Lotan, R., Belloni, P. N., Tressler, R. J., Lotan, D., Xu, X.-C., and Nicolson, G. L. Expression of galectins on microvessel endothelial cells and their involvement in tumor cell adhesion. *Glycoconj. J.*, *11*: 462–468, 1994.
- Bresalier, R. S., Byrd, J. C., Wang, L., and Raz, A. Colon cancer mucin: a new ligand for the  $\beta$ -galactoside-binding protein galectin-3. *Cancer Res.*, *56*: 4354–4357, 1996.
- Duffy, M. J. CA 15-3 and related mucins as circulating markers in breast cancer. *Ann. Clin. Biochem.*, *36*: 579–586, 1999.
- Glinsky, G. V. Antiadhesion cancer therapy. *Cancer Metastasis Rev.*, *17*: 177–185, 1998.
- Kerbel, R. S., St. Croix, B., Rak, J., and Graham, C. H. Is there a role for antiadhesives as chemosensitizers in the treatment of solid tumors by chemotherapy? *Bull. Inst. Pasteur*, *92*: 248–256, 1995.
- Kerbel, R. S., St. Croix, B., Florenes, V. A., and Rak, J. Induction and reversal of cell adhesion-dependent multicellular drug resistance in solid breast tumors. *Hum. Cell*, *9*: 257–264, 1996.
- Inohara, H., and Raz, A. Effects of natural complex carbohydrate (citrus pectin) on murine melanoma cell properties related to galectin-3 functions. *Glycoconj. J.*, *11*: 527–532, 1994.

# Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

## The Role of Thomsen-Friedenreich Antigen in Adhesion of Human Breast and Prostate Cancer Cells to the Endothelium

Vladislav V. Glinsky, Gennadi V. Glinsky, Kate Rittenhouse-Olson, et al.

*Cancer Res* 2001;61:4851-4857.

**Updated version** Access the most recent version of this article at:  
<http://cancerres.aacrjournals.org/content/61/12/4851>

**Cited articles** This article cites 44 articles, 13 of which you can access for free at:  
<http://cancerres.aacrjournals.org/content/61/12/4851.full#ref-list-1>

**Citing articles** This article has been cited by 26 HighWire-hosted articles. Access the articles at:  
<http://cancerres.aacrjournals.org/content/61/12/4851.full#related-urls>

**E-mail alerts** [Sign up to receive free email-alerts](#) related to this article or journal.

**Reprints and Subscriptions** To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at [pubs@aacr.org](mailto:pubs@aacr.org).

**Permissions** To request permission to re-use all or part of this article, use this link  
<http://cancerres.aacrjournals.org/content/61/12/4851>.  
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.