

Serum Galectin-2, -4, and -8 Are Greatly Increased in Colon and Breast Cancer Patients and Promote Cancer Cell Adhesion to Blood Vascular Endothelium

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Abstract

Purpose: Adhesion of disseminating tumor cells to the blood vascular endothelium is a pivotal step in metastasis. Previous investigations have shown that galectin-3 concentrations are increased in the bloodstream of patients with cancer and that galectin-3 promotes adhesion of disseminating tumor cells to vascular endothelium *in vitro* and experimental metastasis *in vivo*. This study determined the levels of galectin-1, -2, -3, -4, -8, and -9 in the sera of healthy people and patients with colon and breast cancer and assessed the influence of these galectins on cancer-endothelium adhesion.

Experimental Design: Serum galectins and auto-anti-MUC1 antibodies were assessed using ELISA and mucin protein (MUC1) glycan microarrays, and cancer-endothelium adhesion was determined using monolayers of human microvascular lung endothelial cells.

Results: The levels of serum galectin-2, -3, -4, and -8 were significantly increased up to 31-fold in patients with cancer and, in particular, those with metastases. As previously shown for galectin-3, the presence of these galectins enhances cancer-endothelium adhesion by interaction with the Thomsen-Friedenreich (TF; Gal β 1,3GalNAc α -) disaccharide on cancer-associated MUC1. This causes MUC1 cell surface polarization, thus exposing underlying adhesion molecules that promote cancer-endothelium adhesion. Elevated circulating galectin-2 levels were associated with increased mortality in patients with colorectal cancer, but this association was suppressed when anti-MUC1 antibodies with specificity for the TF epitope of MUC1 were also present in the circulation.

Conclusions: Increased circulation of several members of the galectin family is common in patients with cancer and these may, like circulating galectin-3, also be involved in metastasis promotion. *Clin Cancer Res*; 17(22); 7035–46. ©2011 AACR.

Introduction

Adhesion of disseminating tumor cells to the blood vascular endothelium is a crucial step in cancer metastasis and is regulated by various cell surface adhesion molecules/ligands on cancer cells as well as on vascular endothelial cells (1).

Galectins form a family of 15 mammalian galactoside-binding proteins expressed by many types of human cells. They are synthesized in the cell cytoplasm from where they can be transported into the cell nucleus, possibly via both active transport and passive diffusion (2), or secreted through a nonclassical pathway (3). Cytoplasmic galectins are involved in the regulation of cellular apoptosis (4, 5). The presence of galectin-1 and -3 in the cell nucleus promotes mRNA splicing (6). The cell surface-associated galectins act as cell adhesion molecules and promote cell-cell and cell-matrix interactions during cancer development and progression (7, 8).

Earlier studies have reported that the concentration of galectin-3 is significantly increased in the sera of patients with colorectal, lung (9), bladder (10), head and neck cancers (11), and melanoma (12–14). Our recent studies have revealed that an increased circulation of galectin-3 promotes cancer cell blood-borne metastasis in nude mice (15). This effect is a result of the galectin-3 interaction with the oncofetal Thomsen-Friedenreich (TF; Gal β 1,3GalNAc) disaccharide expressed by the cancer-associated transmembrane mucin protein MUC1 (16). The galectin-3-TF/MUC1

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Translational Relevance

Adhesion of invaded tumor cells to the blood vascular endothelium is a pivotal step in metastasis. This study revealed that the levels of several galectin members were greatly increased in the sera of patients with colon and breast cancer and that galectins promote cancer cell adhesion to blood vascular endothelium *in vitro* by interaction with cancer-associated Thomsen-Friedenreich (TF)/mucin protein 1 (MUC1). Elevated circulating galectin-2 levels are a marker of poor prognosis in colon cancer, whereas higher levels of circulating auto-anti-MUC1 antibodies with specificity for the TF epitope of MUC1 protected against the poor prognosis associated with increased circulating galectin-2. Targeting the actions of circulating galectins may therefore represent a promising therapeutic approach to reduce metastasis.

interaction induces MUC1 cell surface polarization and exposure of the underlying adhesion molecules that increases cancer cell heterotypic adhesion to vascular endothelium and cancer cell homotypic aggregation to form microtumor emboli (17).

There is limited information about the expression or role of the other galectin members in the circulation. Subcutaneous injection of mouse breast tumor NeuTL cells to FVB/N mice has been shown to increase plasma levels of galectin-1 (18). Serum galectin-1 level is increased in thyroid cancer (11) but not in head and neck squamous cell carcinomas (19). As members of the galectin family share similar carbohydrate-binding properties and many are often found to be coexpressed in the same cell types and tissues, we speculated that other galectin members may also be altered in the bloodstream of patients with cancer and, like galectin-3, may also be involved in cancer cell adhesion to vascular endothelium in metastasis.

This study shows that the concentrations of free circulating galectin-2, -3, -4, and -8 are all markedly increased in the blood circulation of patients with colon and breast cancer and, in particular, those with metastasis. The presence of these galectins promotes cancer cell adhesion to vascular endothelial cells by interaction with the TF disaccharide on cancer-associated MUC1, an effect that is diminished when anti-MUC1 antibodies against the TF epitope of MUC1 are also present in the circulation, probably as a result of their competitive binding to cancer-associated TF/MUC1.

Materials and Methods

Materials

Recombinant human galectin-1, -2, -3, -4, and -8, all the anti-galectin antibodies and biotinylated anti-galectin antibodies were from R&D Systems. B27.29 anti-MUC1 monoclonal antibody was kindly provided by Dr. Mark Reddish (Biomira Inc.). Biotin-conjugated peanut agglutinin (PNA) was purchased from Vector Laboratories Ltd.

Endo-*N*-acetyl-galactosaminidase (EC 3.2.1.97), *O*-glycanase, was obtained from Prozyme Inc. The Calcein AM cell labeling solution was from Invitrogen and the Non-Enzymatic Cell Dissociation Solution (NECDS) was from Sigma.

Cell culture

Human colon cancer HT29-5F7 cells, kindly provided by Dr. Thecla Lesuffleur [INSERM U560, Lille, France], are enterocyte-like subpopulations of HT29 cells that express mainly MUC1 and MUC5B and were isolated as a consequence of their resistance to 5-fluorouracil (20). Human colon cancer SW620 cells were obtained from the European Cell Culture Collections via the Public Health Laboratory Service (Porton Down, Wiltshire, United Kingdom). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) as described in our previous studies (16). Human umbilical vein endothelial cells (HUVEC) and human microvascular lung endothelial cells (HMVEC-L) were obtained from Lonza and were cultured in endothelial growth media (EGM) and EGM-2 and EGM supplements (EGM Bulletkits and EGM-2 Bulletkits, respectively). MUC1 transfection of HBL-100 human breast epithelial cells with full-length cDNA encoding MUC1 and the subsequent selection of the MUC1-positive transfectant HCA1.7⁺ and the negative revertant HCA1.7⁻ was described previously (21).

Serum samples

Fifty-one serum samples from patients with colorectal cancer, 40 without clinically detectable metastasis (26 males and 14 females) and 11 with liver metastasis (7 males and 4 females), and 40 serum samples from patients with breast cancer (all females) were obtained from Cancer Tissue Bank Research Centre (CTBRC). These serum samples were obtained by CTBRC from patients at time when their primary tumors were removed by surgery at the Royal Liverpool Hospital. The length of survival of these patients in the next 10 years was followed up. Thirty-one serum samples from healthy people (13 male and 19 female) were obtained from Sera Laboratories International. The patients' ages were from 25 to 91 years (mean age: 63) and healthy people from 20 to 51 years (mean: age 37; Supplementary Table S1). Although the healthy controls were younger than the patients, there was no association between age and concentration of any of the galectins (Spearman ρ correlation coefficient: -0.24, -0.21, 0.02, -0.034, and -0.18, with $P = 0.19, 0.27, 0.92, 0.86,$ and 0.29 for galectin-1, -2, -3, -4, and -8, respectively).

Determination of serum galectins

High-binding 96-well plates were coated with anti-galectin antibody at 2.5 $\mu\text{g/mL}$ in coating buffer (15 mmol/L Na_2CO_3 and 17 mmol/L NaHCO_3 , pH = 9.6) overnight at 4°C. The plate was washed with washing buffer (0.05% Tween-20 in PBS) and incubated with blocking buffer (1% bovine serum albumin in PBS) for 1 hour at room temperature. Serum samples or standard recombinant galectins

were introduced to the plates for 2 hours before application of biotinylated anti-galectin antibody (1.25 µg/mL in blocking buffer; Supplementary Table S2) for 1 hour at room temperature. After introduction of ExtrAvidin peroxidase (1:10,000 dilution in blocking buffer) for 1 hour, the plates were developed with SigmaFAST OPD for 10 minutes. The reaction was stopped by adding 4 mol/L sulfuric acid, and the absorbance was read at 492 nm by a microplate reader.

Assessments of galectin binding to TF-expressing glycans

High-binding 96-well plates were coated with 5 µg/mL TF-expressing glycoproteins [Antarctic fish antifreeze glycoprotein (AFG), asialofetuin (ASF), or asialo bovine mucin (ABM)] in coating buffer overnight at 4°C. The plates were washed and incubated with blocking buffer for 1 hour before introduction of recombinant galectins (1 µg/mL) for 2 hours at room temperature. Biotinylated anti-galectin antibodies (1.25 µg/mL in blocking buffer) were introduced for 1 hour at room temperature. After application of ExtrAvidin peroxidase for 1 hour, the plates developed with SigmaFAST OPD, and the absorbance was read at 492 nm as above.

In some experiments, the plates were coated overnight at 4°C with 5 µg/mL ABM pretreated with or without 1.25 U/mL *O*-glycanase for 2 hours at 37°C before application of recombinant galectin (1 µg/mL) and subsequent measurements of TF expression or galectin binding by ELISA as above.

Assessments of the effects of galectins on cancer cell adhesion to endothelial monolayers

HUVECs or HMVEC-Ls were released from the culture flasks by trypsinization and resuspended at 1×10^5 per milliliter in EGM or EGM-2 endothelial culture medium. A total of 1×10^4 cells were applied to white walled, clear bottomed, 96-well plates at 37°C for 2 to 3 days for the formation of tight endothelial monolayers.

HT29-5F7 or SW620 cells detached from culture flasks with NECDS were suspended in serum-free DMEM to 1×10^5 cells/mL and incubated with 10 µL/mL Calcein AM cell labeling solution at 37°C for 30 minutes on a shaking water bed at 80 rpm. The cells were washed with serum-free DMEM and resuspended in fresh serum-free DMEM to 5×10^4 /mL. Ten microliters of 100 µg/mL recombinant galectin was preincubated with 10 µL of 10 µmol/L lactose for 30 minutes before mixing with 960 µL cell suspension for 1 hour at 37°C. One hundred microliter cell suspension was then introduced to the endothelial monolayers with or without the addition of 10 µg/mL (final concentration) anti-TF (TF5) antibody (22) for 30 minutes at 37°C. After washing with PBS, the cell-associated fluorescence was measured by a fluorescence microplate reader.

In some experiments, the HT29-5F7 cells were pretreated with or without 0.02 U/mL *O*-glycanase for 3 hours at 37°C before incubation with each galectin (1 µg/mL) and subsequent analysis of cell adhesion to HMVEC-L monolayers as above.

In some other experiments, the Calcein AM-labeled cancer cells were applied to endothelial monolayers cultured in 24-well plates inserted with glass coverslips. The number of adherent cells at the end of experiments was counted under fluorescence microscope, and the cell adhesion was expressed as number of cells/field of view as previously described (15).

Assessment of MUC1 cell surface localization

HCA1.7⁺ human breast epithelial HBL-100 cells were released from the culture flasks by NECDS and resuspended at 5×10^5 /mL in serum-free DMEM. The cells were incubated with or without 1 µg/mL galectin for 1 hour at 37°C. One hundred fifty microliter cell suspension was applied to a poly-lysine-coated slide for 30 minutes before fixing with 2% paraformaldehyde for 15 minutes at room temperature. The slides were incubated with 5% goat serum in PBS for 30 minutes before application of anti-MUC1 B27.29 (2.5 µg/mL in 1% goat serum/PBS) for 1 hour. After washing, the slides were incubated with Texas Red-conjugated secondary antibody (2.5 µg/mL in 1% normal goat serum) for 1 hour before mounting with Vectorshield Mounting Medium (Vector Laboratories). The slides were then blinded and the localization of cell surface MUC1 was analyzed by the fluorescent microscopy with 25× objective.

MUC1 siRNA knockdown

HT29-5F7 cells were released from the culture flasks by trypsinization and resuspended at 5×10^4 per milliliter in antibiotic-free DMEM. The cell suspension was seeded in 96-well plates and incubated at 37°C overnight. MUC1 siRNA or nontargeting control siRNA (100 nmol/L) was applied at 37°C for 72 hours. The cells were either lysed for protein assessments by immunoblotting with anti-MUC1 antibody (B27.29) or released with NECDS for subsequent cell adhesion assessments.

Determination of auto-anti-MUC1 antibodies in serum

MUC1 peptides were synthesized, *O*-glycosylated *in vitro* using various recombinant glycosyltransferases and purified exactly as previously described (23, 24). The glycopeptides were printed on Schott Nexterion Slide H (Schott AG) on a BioRobotics MicroGrid II spotter (Genomics Solution) with a 0.21-mm pitch using Stealth 3B Micro Spotting Pins (Telechem International ArrayIt Division). The slides were incubated in a humidified hybridization chamber with 75% relative humidity for 1 hour before the unspotted areas of the slides were blocked with 25 mmol/L ethanolamine in 100 mmol/L sodium borate (pH = 8.5) for 1 hour. Serum samples (1:25 serial dilution) or monoclonal antibodies (1 µg/mL) were introduced to the slides in a closed container with gentle agitation for 1 hour. After washing with 0.05% Tween-20/PBS, the slides were incubated with Cy3-conjugated goat anti-human immunoglobulin G (IgG; Fc specific; 1:5,000 dilution in PBS-T) for 1 hour. After washing, the slides were dried and scanned in a ProScanArray HT microarray scanner (PerkinElmer) followed by image analysis with ProScanArray Express 4.0 software (PerkinElmer).

Each spot was done in 4 replicates, and the mean value of relative fluorescence intensity was obtained. For comparison, slides were scanned with identical scanning parameters. Data were analyzed using GraphPad Prism software (23).

Statistical analysis

One-way ANOVA followed by Dunnett or Kruskal–Wallis test for multiple comparisons or the Fishers exact test was used where appropriate.

For assessing the relationship between serum galectin concentrations and mortality risk in patients with cancer, Cox proportional hazards analysis (STATA, version 10.0; StataCorp) was used. The analyses were adjusted for age, gender, and disease stage. Hazard ratios (HR) were used to describe the change of mortality risk when the galectin levels are increased by 2- or 10-fold. $P < 0.05$ from 2-sided tests was considered to be statistically significant.

Results

The concentrations of several galectin members are significantly altered in the sera of patients with colorectal and breast cancer in comparison with healthy people

The concentrations of 6 galectins (galectin-1, -2, -3, -4, -8, and -9) were analyzed in the sera from 31 healthy people, 51 colorectal cancer (40 without detectable metastasis and 11 with liver metastasis), and 40 patients with breast cancer. In comparison with healthy people, the concentrations of 4 galectins, galectin-2, -3, -4, and -8, were significantly increased in patients with both colorectal and breast cancer (Fig. 1A–F). The median concentrations of galectin-2 were 1.9-fold higher ($P < 0.001$) in patients with colorectal cancer and 2.3-fold higher ($P < 0.001$) in those who also had liver metastases (Supplementary Table S3). The galectin-3 concentration was 11.3-fold higher ($P < 0.001$) in patients with colorectal cancer and 31.6-fold higher ($P < 0.001$) in those with metastases. Galectin-4 was 11.1-fold higher ($P < 0.001$) in patients with colorectal cancer and 25.3-fold higher ($P < 0.001$) in those with metastases. Galectin-8 was 1.8-fold higher ($P < 0.01$) in patients with colorectal cancer and 5.6-fold ($P < 0.01$) higher in those with metastases. The levels of serum galectin-2, -3, -4, and -8 were also significantly increased (1.2-, 11.3-, 11.0-, and 1.8-fold higher, respectively) in the serum of patients with breast cancer. The concentration of serum galectin-1 was significantly lower in patients with breast cancer than in healthy people, and galectin-9 was unchanged but both of these galectins showed significant increase in patients with metastatic colon cancer. Spearman correlation analysis showed trends of correlations between cancer stages and the levels of galectin-2, -3, and -8 ($P = 0.15, 0.27, \text{ and } 0.12$, respectively) in colorectal cancer and of galectin-2 and -4 ($P = 0.14$ and 0.12 , respectively) in breast cancer, although none of those reached statistical significance.

As earlier studies have suggested that circulating clotting factors carry both *N*- and *O*-linked glycans and are recog-

nized by some galectins (25, 26), we assessed possible differences between serum and plasma levels of these galectins by spiking freshly obtained blood samples before clotting or addition of heparin and separation of serum or plasma. We found no significant differences in the galectin-1 and -2 levels in the plasma in comparison with serum but galectin-3 and -4 levels were significantly (3.0- and 1.9-fold) higher in plasma than in serum. This suggests that the real circulating concentrations of galectin-3 and -4 for healthy people and in the patients with cancer are higher than those reported here in sera.

Galectins increase cancer cell adhesion to endothelial cells

Our previous investigations have shown that an increased circulation of galectin-3 enhances cancer cell adhesion to vascular endothelial cells in metastasis (15). As the concentrations of galectin-2, -4, and -8 were also shown to be increased in the circulation of patients with cancer and in particularly those with metastasis, we assessed the effects of those galectin members on cancer cell adhesion to vascular endothelium.

Preincubation of human colon cancer HT29-5F7 cells with recombinant galectin-2, -4, or -8 at concentrations similar to those observed in patients with cancer, all induced a dose-dependent increase of HT29-5F7 cell adhesion to HUVEC monolayers (Fig. 2A). At 1 $\mu\text{g/mL}$, galectin-2, -4, and -8 caused $168\% \pm 73\%$ ($P < 0.001$; ANOVA and Dunnett test), $189\% \pm 32\%$ ($P < 0.001$), and $180\% \pm 21\%$ ($P < 0.001$), respectively, increase in cell adhesion. The effects of these galectins on cancer cell adhesion were completely abolished by the presence of 10 $\mu\text{mol/L}$ lactose (Fig. 2B). Furthermore, each galectin at 1 $\mu\text{g/mL}$ also induced significant increase of adhesion to HMVEC-Ls of HT29-5F7 as well as SW620 cells but not HT29 cells (Fig. 2C). HT29-5F7 cells were a subpopulation of the parental HT29 cells selected for their resistance to 5-fluorouracil and have been shown previously to have higher MUC1 expression than the parent HT29 cells (17, 20).

The expression of TF-expressing MUC1 is important in galectin-mediated cancer-endothelium adhesion

Our previous studies have shown that galectin-3 increases cancer cell adhesion to endothelial cells by interaction with the TF disaccharide on cancer-associated MUC1 (16), the discovery that galectin -2, -4, and -8 could each induce cancer cell adhesion to endothelial cells led us to investigate whether these galectin effects were also, like that of galectin-3, associated with interaction of the galectins with the TF disaccharide on MUC1.

To test this, we first determined by direct galectin ELISA whether these galectin members recognized the TF disaccharides. It was found that all these galectins recognized the TF-expressing AFG, ASF, and ABM, although with different binding affinities (Fig. 3A). ABM is the strongest ligand for galectin-4, whereas ASF is the strongest ligand for galectin-2. Mucin proteins, such as ABM, are known to carry TF as well as many other carbohydrate structures (27). Treatment of

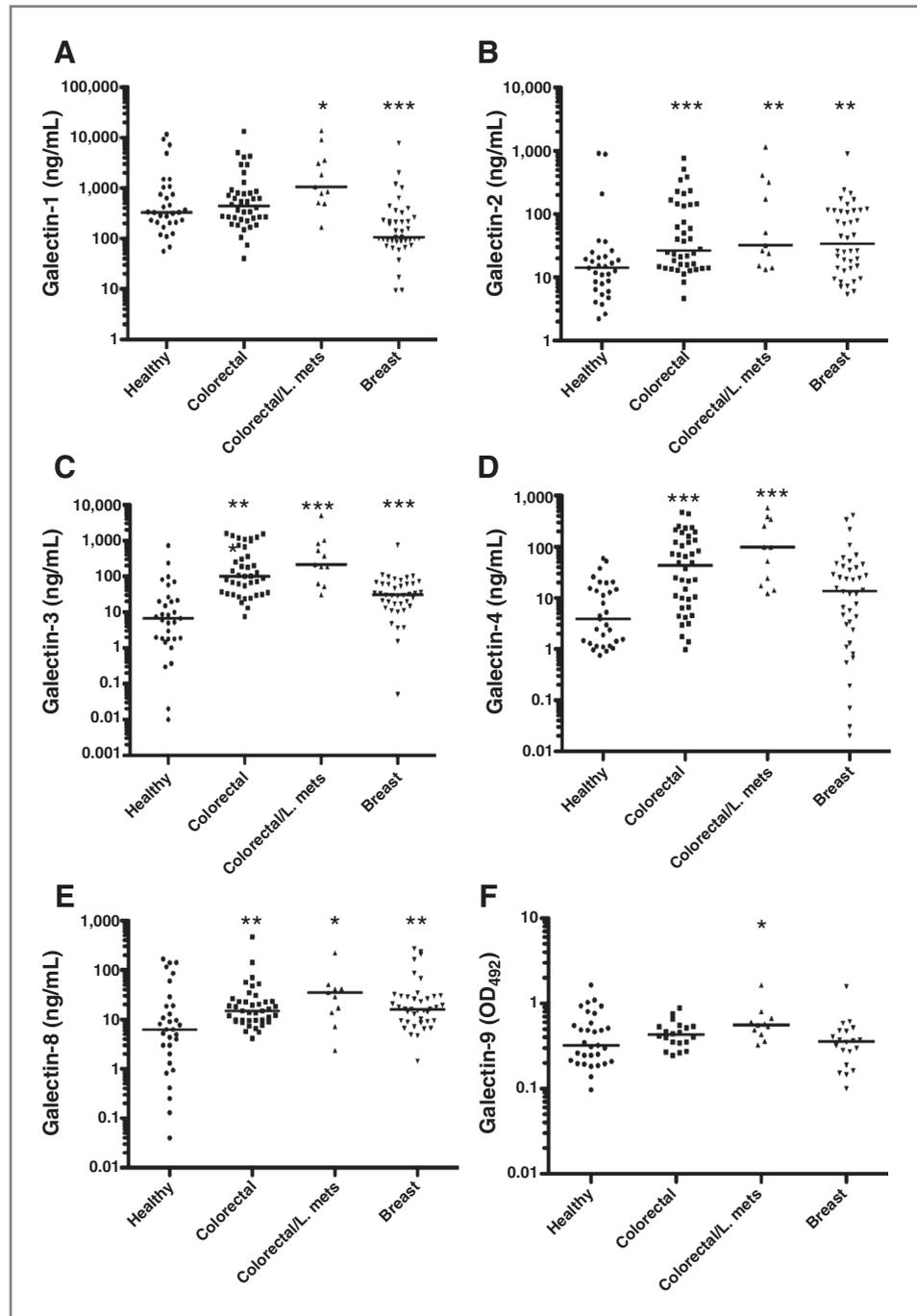


Figure 1. Concentrations of circulating galectins in the sera of patients with colorectal and breast cancer and healthy people. Serum galectin-1 (A), -2 (B), -3 (C), -4 (D), -8 (E), and -9 (F) levels were assessed by individual galectin ELISA and calculated from standard curves derived using recombinant galectins run in parallel in each assessment (galectin-9 level is shown as optical density reading, as no recombinant galectin-9 is commercially available as a standard; note different y scales are used in the figures). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. L. mets, liver metastasis; OD, optical density.

ABM with *O*-glycanase specific for removal of unsubstituted TF caused 4.2-fold reduction of TF expression (Fig. 3B) and markedly reduced the binding (by 50% to 80%) of all the galectin members (Fig. 3C). These results indicate binding of all those galectin members to terminal TF disaccharides.

To assess any involvement of cancer-associated TF disaccharide in galectin-mediated cell adhesion, we pretreated the HT29-5F7 cells with *O*-glycanase and assessed the influence of this on galectin-mediated cell adhesion. *O*-Glycanase treatment of HT29-5F7 cells resulted in 70%

reduction of cell surface TF expression when assessed by TF-binding PNA analyzed by flow cytometry (Fig. 3D). Removal of the cell surface TF by *O*-glycanase treatment markedly reduced HT29-5F7 cell adhesion to HMVEC-Ls induced by each of these galectin members (Fig. 3E). This suggests that the terminal TF disaccharide on cancer cell surface is involved in galectin-mediated cancer cell adhesion to endothelium.

Having shown the involvement of cancer-associated TF in galectin-mediated cell adhesion, we then assessed the role

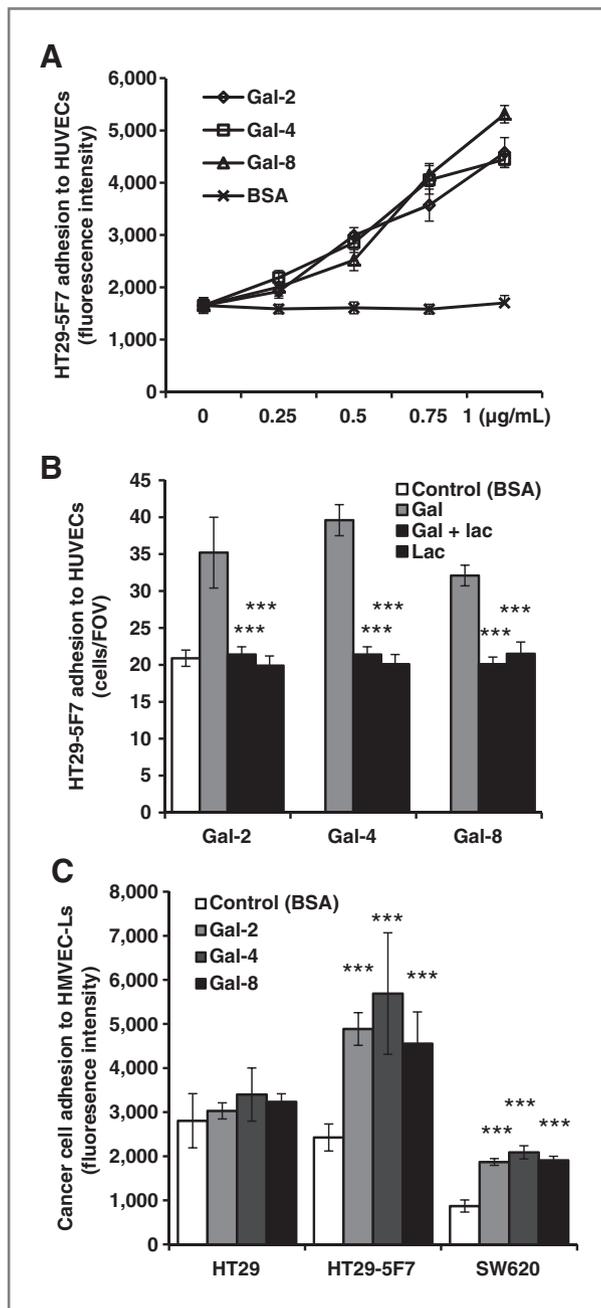


Figure 2. Galectins increase human colon cancer cell adhesion to monolayers of macro- and micro-vascular endothelial cells. **A**, the presence of recombinant galectin-2, -4, or -8 each induced a dose-dependent increase of HT29-5F7 cell adhesion to monolayers of HUVECs. Data are expressed as mean \pm SD of triplicate determinations of 2 independent experiments. **B**, the presence of galectin inhibitors abolishes galectin-induced HT29-5F7 cell adhesion to HUVECs. Data are expressed as mean \pm SD of 10 randomly selected field of view (FOV) of 2 independent experiments. **C**, the presence of each galectins induces cell adhesion to HMVEC-Ls of HT29-5F7 and SW620 cells but not parent HT29 cells. Data are expressed as mean \pm SD of triplicate determinations of 2 independent experiments. ***, $P < 0.001$.

of MUC1 in galectin-mediated cell adhesion. It was found that treatment of HT29-5F7 cells with MUC1 siRNA caused 87% reduction of MUC1 expression (Fig. 4A) and this significantly attenuated the increased cell adhesion to HMVEC-Ls mediated by each of the galectins (Fig. 4B). Together, these results indicate that the MUC1-associated TF contributes to galectin-mediated cancer-endothelium adhesion. This conclusion is further supported by the observations that the presence of each of these galectins induced significant increase of adhesion to HMVEC-Ls of the MUC1 positively transfected (also TF expressing; ref. 15) HCA1.7⁺ cells, but not the MUC1-negative revertants HCA1.7⁻, of human breast epithelial HBL-100 cells (Fig. 4C).

Galectin cell surface binding induces change in MUC1 cell surface localization

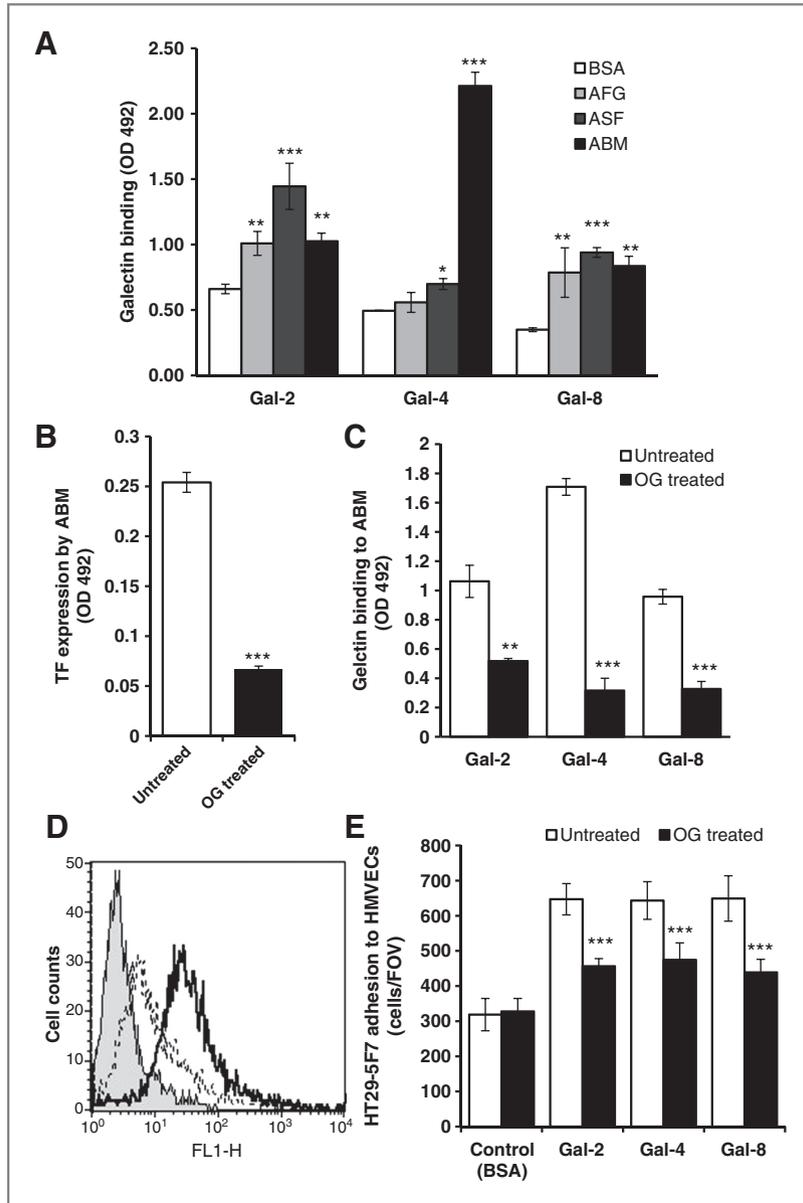
As the galectin-3-mediated cancer cell adhesion is associated with change in MUC1 cell surface localization and exposure of the smaller cell surface adhesion molecules (15), we then analyzed whether the interaction between TF/MUC1 and galectin-2, -4, and -8 also induced changes in MUC1 cell surface localization. Treatment of the HCA1.7⁺ cells with each of these galectin members (1 μ g/mL) resulted in a significant increase in the proportion of cells showing discontinuous MUC1 cell surface localization in comparison with the cells treated with control bovine serum albumin (Fig. 4D and Supplementary Table S4). Thus, as previously shown for galectin-3, the influence of these galectins on cancer cell adhesion is associated with polarization of MUC1 cell surface localization, which allows exposure of the underlying adhesion molecules.

Increased circulation of galectin-2 correlates with increased mortality risk in patients with colorectal cancer

The discovery that several galectin members are all increased in the circulation of patients with colon and breast cancer and that the presence of these galectins enhances cancer cell adhesion to vascular endothelium led us to investigate whether the elevated levels of these galectins in the circulation have direct links with patient's survival.

To test this hypothesis, we used a Cox regression model to analyze the relationship between circulating galectin levels at the time of surgical removal of the primary tumors and subsequent 10-year survival for patients with colorectal and breast cancer. This survival regression analysis, which was adjusted for age, gender, and disease stage, showed no significant correlation between the serum concentrations of galectin-1, -3, -4, and -8 and patients survival for patients with either colorectal or breast cancer (Table 1). However, the increased level of circulating galectin-2 correlated with a significantly increased mortality in patients with colorectal cancer. A 2-fold increase of serum galectin-2 level was associated with an 18% increase of mortality risk, whereas a 10-fold increase was associated with a 75% increased risk of 10-year mortality ($P = 0.013$).

Figure 3. Galectin-mediated cancer cell adhesion is associated with galectin binding to TF disaccharide on cancer cells. **A**, direct galectin ELISA shows binding of recombinant galectin-2, -4, and -8 to TF-expressing ABM, ASF, and AFG. Data are expressed as mean \pm SD of triplicate determinations. **B**, O-glycanase treatment of ABM reduces the expression of TF. ABM (5 μ g/mL) was treated with or without 1.25 U/mL O-glycanase (OG) before it was used as substrate for the detection of TF expression by ELISA with TF-binding PNA. Data are expressed as mean \pm SD of triplicate determinations. **C**, removal of the TF on ABM by O-glycanase reduces galectin binding. Data are expressed as mean \pm SD of triplicate determinations. **D**, treatment of HT29-5F7 cells with O-glycanase reduces cell surface TF expression. The cell surface TF expression was assessed with TF-binding PNA and analyzed by flow cytometry after treatment of the cells with O-glycanase (solid line histogram, without O-glycanase treatment; dotted line histogram, after O-glycanase treatment; shaded histogram, isotype control). A shift of the histogram to the left after O-glycanase treatment indicates a reduction of the cell surface TF expression. **E**, removal of the cell surface TF by O-glycanase reduces galectin-mediated HT29-5F7 cell adhesion to HMVEC-Ls. Data are expressed as mean \pm SD of absolute number of cells per FOV from at least 10 randomly selected FOVs of triplicate determinations of 2 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. OD, optical density.



Serum galectin-2-associated reduction in survival is reduced by coexistence of autoantibodies against TF/MUC1

It is known that various auto-anti-MUC1 antibodies exist in the blood circulation of patients with cancer (23). As the influence of circulating galectins on cancer cell adhesion to vascular endothelium is attributed by their interaction with cancer-associated TF/MUC1, we further speculated that the presence of auto-anti-MUC1 antibodies specifically against the TF epitope of MUC1 may produce a preventive effect on galectin-mediated adhesion hence metastasizing as a result of their competitive binding to TF/MUC1 on disseminating tumor cells in the bloodstream.

To test this possibility, we determined the levels of auto-anti-MUC1 antibodies against the TF as well as sialyl-TF

epitope of MUC1 in the sera of 51 patients with colorectal cancer using an O-glycopeptide microarray (23). The serum levels of the auto-anti-MUC1 antibodies against its TF epitope and sialyl-TF epitope in these patients ranged from 0 to 19,444 and from 28 to 20,763 units, respectively (Supplementary Fig. S1A and S1B). When the patient samples were divided into 2 subgroups with equal numbers of high and low (above and below the median 1,290 units) levels of autoantibodies against TF/MUC1, the galectin-2-associated increase of mortality risk remained significant only in the low level of auto-anti-TF/MUC1 group but not in the high level of auto-anti-TF/MUC1 group (Table 2). On the other hand, when the patient samples were divided into 2 subgroups with equal numbers of high and low (above and below the median 2,055 units) levels of autoantibodies

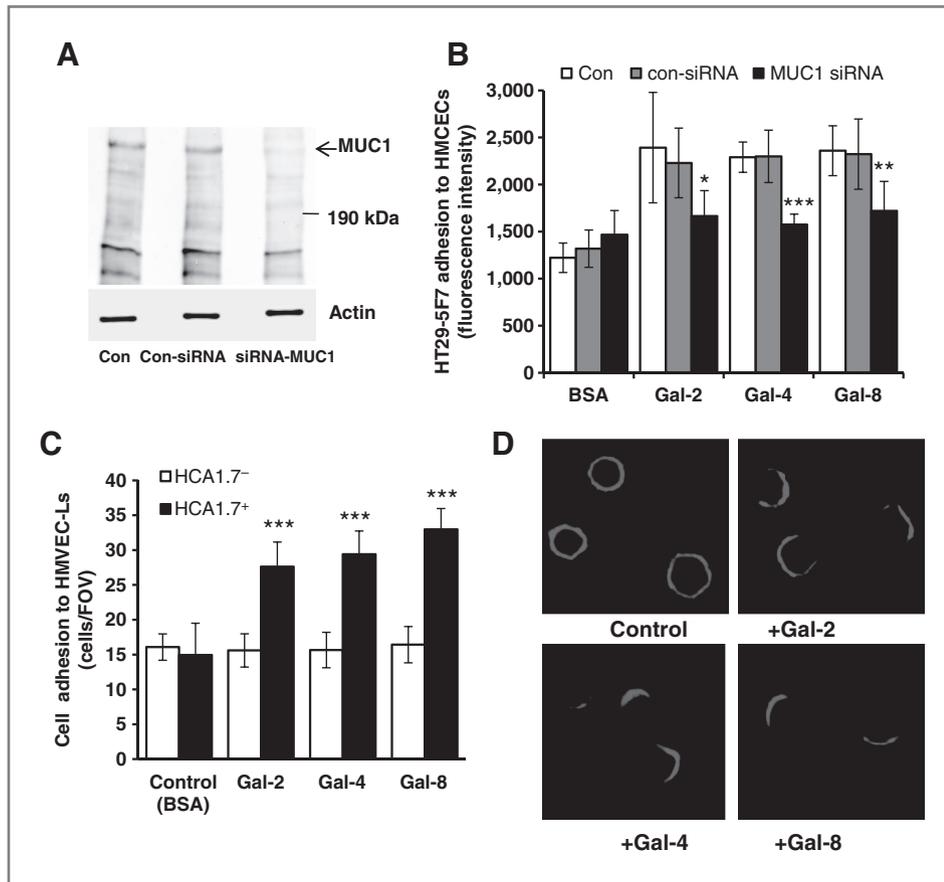


Figure 4. Galectin-mediated cancer cell adhesion is associated with binding of the galectins to MUC1. A, suppression of MUC1 expression by siRNA. HT29-5F7 cells were treated with or without MUC1 siRNA or control siRNA before the expressions of MUC1 or β -actin were assessed by anti-MUC1 (B27.29) or anti- β -actin immunoblotting. Representative blots are shown. B, MUC1 siRNA suppression abolishes galectin-mediated cell adhesion to HMVEC-Ls. Data are expressed as mean \pm SD of triplicate determinations of 2 independent experiments. C, galectins induce adhesion to HMVEC-Ls of MUC1 positively transfected but not negatively transfected HBL-100 cells. Data are expressed as mean \pm SD of triplicate determinations of 2 independent experiments. D, representative images of MUC1 cell surface localization of HCA1.7⁺ cells with or without treatment of each galectin (1 μ g/mL). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Con, control; con-siRNA, control siRNA.

against the sialylated TF/MUC1, the galectin-2-associated increase of mortality risk remained significant in both groups ($P = 0.043$ in the high group and 0.004 in the low group). No correlation was seen between the levels of auto-anti-TF/MUC1 and anti-sialyl-TF/MUC1 antibodies themselves and mortality risk (not shown). It is known that sialylation of the TF residue of MUC1 reduces galectin binding to TF/MUC1 (16). Collectively, these discoveries suggest that the presence of antibodies against the TF-glycosylated MUC1 epitope prevents the interaction of circulating galectins with cancer cell-associated TF/MUC1 and hence blocks their effect on cancer-endothelium adhesion in metastasis as a result of competitive binding to cancer-associated TF/MUC1. This conclusion is supported by the demonstration that the presence of a human anti-TF antibody (TF5) resulted in significant inhibition of the increase of HT29-5F7 cell adhesion to HMVEC-Ls induced by each of these galectins (Supplementary Fig. S1C).

Discussion

This study shows that the concentrations of serum galectin-2, -3, -4, and -8 levels are all significantly higher in patients with colon and breast cancer than in healthy people. Patients with metastasis also have higher levels of circulating galectins than those without metastasis. The

presence of these galectins promotes cancer cell adhesion to vascular endothelium *in vitro* as a result of the galectin interactions with cancer-associated TF/MUC1. Such a metastasis-promoting effect of circulating galectins is supported by a direct association between increased circulating galectin-2 level and increased mortality risk in patients with colorectal cancer, an association that is diminished when a subgroup of auto-anti-MUC1 antibodies with specificity for TF-glycosylated MUC1 is also present in the circulation, as a result of their competitive binding to cancer-associated TF/MUC1.

The mechanism for the increased circulating galectin in patients with cancer is unclear. Members of the galectin family are expressed by many types of human cells including epithelial, endothelial, and immune cells. Earlier reports have shown that the plasma or serum levels of galectin-3 (9), -1, and -4 (28) were significantly reduced following surgical removal of the primary tumors in patients with colorectal cancer. This indicates that tumor cells may make significant contributions to the increased circulation of those galectin members. On the other hand, cellular expressions of galectin-8 and -4 have been reported to be lower in colorectal cancer than in healthy colonic epithelium (29, 30). It seems likely, therefore, that other cells, for example, stromal or immune cells, may be the major contributor to the

Table 1. Relationship between serum galectin levels and 10-year mortality risk in patients with colon and breast cancer

	HR	SE	P	95% CI
<i>Colorectal cancer (n = 51)</i>				
Gal-1				
2-fold	0.96	0.074	0.60	0.83–1.11
10-fold	0.88	0.22	0.60	0.53–1.44
Gal-2				
2-fold	1.18	0.81	0.013	1.04–1.36
10-fold	1.76	0.40	0.013	1.12–2.75
Gal-3				
2-fold	1.00	0.07	0.97	0.87–1.14
10-fold	0.99	0.23	0.97	0.63–1.57
Gal-4				
2-fold	0.89	0.54	0.06	0.79–1.00
10-fold	0.68	0.14	0.06	0.46–1.02
Gal-8				
2-fold	1.01	0.98	0.94	0.82–1.22
10-fold	1.03	0.33	0.94	0.54–1.93
<i>Breast cancer (n = 40)</i>				
Gal-1				
2-fold	1.13	0.11	0.19	0.94–1.39
10-fold	1.54	0.51	0.19	0.81–2.96
Gal-2				
2-fold	1.02	0.11	0.82	0.83–1.26
10-fold	1.08	0.38	0.82	0.55–2.14
Gal-3				
2-fold	1.03	0.11	0.82	0.83–1.27
10-fold	1.08	0.40	0.82	0.53–2.22
Gal-4				
2-fold	1.02	0.079	0.84	0.87–1.18
10-fold	1.05	0.27	0.84	0.64–1.75
Gal-8				
2-fold	0.90	0.13	0.45	0.68–1.18
10-fold	0.70	0.33	0.45	0.28–1.76

NOTE: Cox hazard analysis of the serum galectin levels and patients' survival within 10 years after primary tumor removal was conducted in 51 patients with colorectal and 40 patients with breast cancer. The HRs describe the change of mortality risk when the galectin levels are increased by 2- or 10-fold.

increased circulation of these galectins, in particularly that of galectin-8 and -4. This is in keeping with a recent report showing lack of correlation between galectin-3 serum level and corresponding cancer tissues in thyroid cancer (31).

Several galectin members, for example, galectin-1 and -3, are overexpressed in the tissues surrounding tumors. In colorectal cancer, galectin-1 expression is stronger in the peritumor stromal cells than in the tumors (32). It is possible therefore that the peritumor stromal cells may make important contribution to the increased circulation of galectins in cancer. All immune cells including monocytes, macrophages, and lymphocytes express galectins. The expressions of galectins in immune cells are heavily influenced by inflammatory regulators and also by dif-

ferentiation (33) and activation (34). When stimulated with cytokine granulocyte macrophage colony-stimulating factor (GM-CSF), monocytes have shown to secrete more galectin-3 in cell culture condition (35). Many proinflammatory cytokines, such as TNF- α , interleukins 1 and 8, and GM-CSF, are upregulated in cancerous conditions (1), and their presence may cause the immune cells to secrete more galectins into the bloodstream. Thus, the increased circulation of the members of galectins in patients with cancer has likely come from the peritumor stromal tissues, the immune cells, as well as the tumor cells themselves.

Interestingly, although the serum concentrations of several galectin members are shown, in this study, to be higher in patients with colon cancer and all of these galectins at

Table 2. Influence of the presence of autoantibodies against TF and sialyl-TF epitope of MUC1 on serum galectin-2–associated mortality risk in colorectal cancer

	HR	SE	P	95% CI
Anti-TF/MUC1				
High (n = 26)	1.75	0.87	0.26	0.67–4.63
Low (n = 25)	2.15	0.73	0.024	1.10–4.17
Anti-sialyl-TF/MUC1				
High (n = 26)	2.06	0.73	0.043	1.02–4.15
Low (n = 25)	3.01	1.13	0.004	1.44–6.31

NOTE: The serum auto-anti-MUC1 antibodies against the TF and sialyl-TF epitopes of MUC1 in the 51 patients with colorectal cancer were assessed by glycan microarray coated with 20 $\mu\text{mol/L}$ MUC1 glycopeptides (60 mer) as described in the Materials and Methods section. The serum samples were then divided into 2 groups of above and below the median concentrations of either anti-TF/MUC1 or anti-sialyl-TF/MUC1 antibody. The serum galectin-2–associated mortality risk in patients' survival in the 10-year period was then analyzed by Cox hazards analysis.

pathologic concentrations increase cancer-endothelium adhesion *in vitro*, only the increased level of circulating galectin-2 shows a direct correlation with increased mortality risk in the patients studied here. Although it is possible that the sample size of this study is not large enough to elucidate all the potential relationship of these galectins with survival, the effects of some galectin members on cancer-endothelium adhesion and metastasis are highly likely to be influenced by the presence of different galectin-binding ligands or antibodies in the circulation. Members of the galectin family have shown to bind serum proteins with very different affinities. For example, serum IgGA1 binds strongly to galectin-1 (36), whereas a haptoglobin-related serum glycoprotein (37) and the 90K/Mac-2bp (38) are serum ligands of galectin-3. A systematic analysis of the galectin-binding proteins in human serum using galectin affinity purification followed by electrophoresis and mass spectrometry has shown that galectin-3, -8, and -9 recognize a much broader range of serum ligands, whereas galectin-2, -4, and -7 show binding to only trace or no serum ligands (39). Thus, the influence on metastasis and patient survival of circulating galectin-2 shown in this study may reflect the relative lack of circulating competitive ligands for this galectin.

It is not clear why the increased galectin-2 level in patients with breast cancer was not found to be associated with increased mortality risk as in colorectal cancer. The increase of circulating galectin-2 in patients with breast cancer was less marked than in colorectal cancer. Further studies conducted in larger patient cohorts may clarify the role of galectin-2 in breast cancer. In contrast to the findings here, one recent study found no increase in plasma galectin-2 concentration in colorectal cancer (28). It is possible that the different use of the testing antibodies in that study might contribute to this discrepancy.

The discovery of a protective effect of circulating autoantibodies against specifically the TF epitope of MUC1 on galectin-mediated metastasis is very interesting. This may provide some explanation for reported inconsistencies in

the relationship between the level of general anti-MUC1 antibodies and cancer survival. The levels of auto-anti-MUC1 antibodies have shown to correlate with survival in breast (40) and gastric (41) cancer but not in colorectal cancer (42). It now seems likely that there is a complex relationship between circulating galectin concentration, the presence of circulating autoantibodies against TF-glycosylated MUC1, and cancer survival.

The results presented here also imply that the low efficacy of many of the MUC1-associated immunotherapeutic approaches (either antibody based or peptide vaccine based; ref. 43) may be partly due to the varying glycosylation of MUC1. A more selective targeting of TF-glycosylated MUC1 might be more effective.

Galectins bind to various galactoside-terminal carbohydrate structures that are expressed by various human cells including different immune cells. Galectins are important immune regulators and regulate cell activation, apoptosis, adhesion, migration, and chemotaxis (44–47). An increased circulation of several galectin members in patients with cancer may alter immune surveillance, which, in turn, could contribute to cancer progression and metastasis. Although the concentrations of serum galectins in patients with cancer shown in this study are lower than those typically used in the *in vitro* galectin studies for immune cells (usually at concentrations >10–20 $\mu\text{g/mL}$), galectin binding can induce ligand clustering which, in the case of galectin-3, can enhance the galectin-binding affinity by as much as 10,000-fold (48). Functionally, important galectin concentrations may therefore be achieved in the microenvironment and produce a significant influence on immune surveillance in patients with cancer.

Watanabe and colleagues have recently reported a similar increase of circulating galectin-3 and -4 in patients with colorectal cancer (28). It is noted that the Watanabe study also reported a significant increase of plasma galectin-1 level in patients with colorectal cancer, whereas the present study showed a significant increase of serum galectin-1 in patients with colon cancer that only have liver metastasis. It is

possible that the use of plasma samples in the Watanabe study and the use of serum samples in this study may account for this discrepancy as a consequence of some binding of galectins to activated clotting factors.

Thus, increased circulation of members of the galectin family is a common feature in patients with cancer and may, like that of circulating galectin-3, also promote disseminating tumor cell adhesion to blood vascular endothelium in metastasis as a result of their interactions with the cancer-associated TF/MUC1. Such a metastasis-promoting effect of circulating galectins is, however, prevented when a subgroup of auto-anti-MUC1 antibodies against the TF epitope of MUC1 is also present in the blood circulation as a result of their competitive binding to the cancer-associated TF/MUC1. Elevated circulating galectin-2 concentrations are a marker of poor prognosis in colorectal cancer, whereas higher levels of circulating auto-anti-MUC1 antibodies with specificity for the TF epitope of MUC1 abolished circulating galectin-2-associated poor prognosis. This implies that circulating galectins may represent promising

therapeutic targets for the development of effective agents to reduce metastasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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