Galectin-3 Interaction with Thomsen-Friedenreich Disaccharide on Cancer-associated MUC1 Causes Increased Cancer Cell Endothelial Adhesion*

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Patients with metastatic cancer commonly have increased serum galectin-3 concentrations, but it is not known whether this has any functional implications for cancer progression. We report that MUC1, a large transmembrane mucin protein that is overexpressed and aberrantly glycosylated in epithelial cancer, is a natural ligand for galectin-3. Recombinant galectin-3 at concentrations (0.2–1.0 μg/ml) similar to those found in the sera of patients with metastatic cancer increased adhesion of MUC1-expressing human breast (ZR-75-1) and colon (HT29-5F7) cancer cells to human umbilical vein endothelial cells (HUVEC) by 111% (111 ± 21%, mean ± S.D.) and 93% (93 ± 17%), respectively. Recombinant galectin-3 also increased adhesion to HUVEC of MUC1 transfected HCA1.7+ human breast epithelial cells that express MUC1 bearing the oncofetal Thomsen-Friedenreich antigen (Galβ1,3GalNAc-α (TF)) but did not affect adhesion of MUC1-negative HCA1.7- cells. MUC1-transfected, Ras-transformed, canine kidney epithelial-like (MDE9,2+) cells, bearing MUC1 that predominantly carries sia-llyl-TF, only demonstrated an adhesive response to galectin-3 after sialidase pretreatment. Furthermore, galectin-3-mediated adhesion of HCA1.7+ to HUVEC was reduced by O-glycanase pretreatment of the cells to remove TF. Recombinant galectin-3 caused focal disappearance of cell surface MUC1 in HCA1.7+ cells, suggesting clustering of MUC1. Co-incubation with antibodies against E-Selectin or CD44H, but not integrin-β1, ICAM-1 or VCAM-1, largely abolished the epithelial cell adhesion to HUVEC induced by galectin-3. Thus, galectin-3, by interacting with cancer-associated MUC1 via TF, promotes cancer cell adhesion to endothelium by revealing epithelial adhesion molecules that are otherwise concealed by MUC1. This suggests a critical role for circulating galectin-3 in cancer metastasis and highlights the functional importance of altered cell surface glycosylation in cancer progression.

Galectin-3 is one of 15 known members of the galectin family of naturally occurring galactoside-binding lectins that are expressed intracellularly and extracellularly by many cell types (1). Galectin-3 concentrations are increased up to 5-fold in the sera of patients with breast, gastrointestinal, or lung cancer (2). Moreover, higher galectin-3 concentrations are seen in the sera of patients with metastatic disease than in the sera of patients with localized tumors (2). The source of increased circulating galectin-3 in cancer patients is not clear, but it is probably generated by tumor cells as well as by peritumoral inflammatory and stromal cells (2). It is not known whether this increased circulating galectin-3 has any functional implications for cancer progression.

Cytoplasmic galectin-3 is known to be anti-apoptotic (3), whereas nuclear galectin-3 promotes pre-mRNA splicing (4, 5). Cell surface galectin-3 is involved in various cell-cell and cell-matrix interactions (1, 6, 7) and enhances cancer cell cell adhesion to and invasion through basement membrane by interacting with extracellular matrix proteins such as fibronectin, collagen, or laminin (1, 8, 9). Galectin-3 expressed on the endothelial cell surface has been shown to promote the adhesion of breast cancer cells to endothelium by interaction with cancer-associated Thomsen-Friedenreich (galactose β1,3N-acetylgalactosamine α- (TF))2 antigen expressed by unknown cell surface molecules (10–14). TF antigen is the core 1 structure of mucin-type O-linked glycans, but in its simplest nonsialylated, nonextended form it acts as an oncofetal antigen, and its presence/ expression is increased in malignant and premalignant epithelia (15).

MUC1 (also known as episialin and DF3) is a large (Mr > 250,000) transmembrane mucin protein expressed on the apical surface of most normal secretory epithelia including those in the mammary gland, and the gastrointestinal, respiratory, urinary, and reproductive tracts. The MUC1 extracellular domain consists of variable numbers of 20-amino acid tandem repeat peptides (VNTR) that are rich in serines, threonines, and prolines. These tandem repeat domains are heavily glycosylated

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2 The abbreviations used are: TF antigen, Thomsen-Friedenreich antigen (galactose β1,3N-acetylgalactosamine α-); Tn antigen, N-acetylgalactosamine α-; HUVEC, human umbilical vein endothelial cell; ICAM-1, intercellular cell adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1; PBS, phosphate-buffered saline; ANOVA, one way analysis of variance.
with complex O-glycans (16). There are several splice variants of MUC1, and no functional differences between these MUC1 variants are known (17–19).

In epithelial cancer cells, there is increased expression of glycoforms of MUC1 that show reduced expression of complex O-glycans and increased expression of shorter oligosaccharides such as sialic acid substituted (20–22) and unsubstituted TF antigen (21, 23, 24). MUC1 also undergoes a change in its localization to become expressed over the entire surface in epithelial cancer cells (25, 26). MUC1 has been shown to interact via its cytoplasmic domain with important intracellular proteins including β-catenin (27) and p53 (28) and is therefore involved in signal transduction and regulation of apoptosis. Because of its massive size and length, ~250 nm in comparison with ~28 nm for typical cell surface adhesion molecules like liver cell adhesion molecule (29), cell surface MUC1 is also believed to function as an anti-adhesion molecule by masking cell surface adhesion molecules (30). Thus, overexpression of MUC1 inhibits integrin-mediated adhesion of human breast epithelial cells to extracellular matrix proteins in vitro (31), and down-regulation of MUC1 by antisense oligonucleotide increases E-cadherin-mediated cell–cell aggregation of breast cancer cells (32). Capping of MUC1 on the cell surface of human breast cancer cells as a result of the addition of a cross-linking anti-MUC1 antibody exposes cell adhesion molecules and increases adherence of these cells to the extracellular matrix (30).

In this study we show that MUC1 is a novel and natural ligand of endogenous galectin-3 in human colon cancer cells and that recombinant galectin-3, at concentrations similar to those found in the blood of cancer patients, causes a significant increase in adhesion of epithelial cancer cells to endothelium as a consequence of its interaction with TF expressed on MUC1.

**EXPERIMENTAL PROCEDURES**

**Materials**—Anti-MUC1 (B27.29) and anti-STn (B195.3) (33) antibodies were kindly provided by Dr. Mark Reddish (Biomira Inc., Edmonton, Canada). Mouse anti-galectin-3 antibody was obtained from Novocastra (Newcastle upon Tyne, United Kingdom). Human anti-TF antibody (TF5) was kindly provided by Dr. Bo Jansson (BioInvent Therapeutic, Lund, Sweden) (34). Antibodies against E-Selectin, ICAM-1, VCAM-1, integrin β1, and CD44H were from R & D Systems Europe Ltd (Abingdon, United Kingdom). Peroxidase-conjugated peanut lectin (PNA) and mushroom lectin (ABL), biotin-conjugated jacalin (JAC), Maackia amurensis (MAL-II), and Griffonia simplicifolia lectin (GSL) were purchased from Vector Laboratories Ltd. (Peterborough, United Kingdom). *Arthrobacter ureafaciens* sialidase (EC 3.2.1.18), *Streptococcus pneumoniae* endo-N-acetyl-galactosaminidase (EC 3.2.1.97), O-glycanase, and recombinant N-glycosidase (peptide-N-glycosidase F; EC 3.2.2.18) were obtained from Glyko Inc., (Oxford, United Kingdom). The non-enzymatic cell dissociation solution was from Sigma. The Vybrant DIO and Dil cell labeling solutions were from Molecular Probes (Eugene, OR).

**Cell Lines**—The HT-29 human colon cancer cell line was obtained from the European Cell Culture Collection via the Public Health Laboratory Service (Porton Down, Wiltshire, United Kingdom). HT29-SF7 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 (35). The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 4 mM glutamine at 37 °C in a humidified atmosphere of 5% CO₂. ZR-75-1 human breast cancer cells were kindly provided by Professor David Fernig, School of Biological Science, University of Liverpool and were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 0.1 μg/ml estradiol, 100 unit/ml penicillin, 100 μg/ml streptomycin, and 4 mM glutamine. Human umbilical vein endothelial cells (HUVEC) were obtained from American Type Culture Collection and were cultured in F12K medium supplemented with 0.1 mg/ml heparin, 0.03 mg/ml endothelial cell growth supplement (Sigma), and 10% fetal calf serum at 37 °C. 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− cells were as previously described (26). MUC1 transfection of Ras-transformed Madin-Darby canine kidney epithelial-like MDCK-Ras-e cells with full-length cDNA encoding MUC1 and the subsequent selection of the MUC1 positive transfectant MDE9.2+ and the negative revertant MDE9.2− were also as previously described (26).

**Production of Human Recombinant Galectin-3**—Recombinant human galectin-3 was produced in *Escherichia coli* using pET21a expression vector, which was ligated with a cDNA sequence encoding for human galectin-3, and affinity-purified using asialofetuin-Sepharose 4B as previously described (36).

**Immunoprecipitation and Immunoblotting**—Subconfluent HT-29 cells were released from the culture plates using a cell scraper (Coster) and lysed on ice in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, plus 2% aprotinin, and 20 μg/ml leupeptin) for 10 min before a brief sonication (30 s). The supernatants of the cell lysates were collected after centrifugation at 100,000 × g for 1 h. After dilution to 1 mg of protein/ml with lysis buffer, 1-ml supernatants were precleared with 50 μl of protein A-agarose for 1 h. After dilution to 1 mg of protein/ml with lysis buffer, 1-ml supernatants were precleared with 50 μl of protein A-agarose for 20 min at 4 °C before incubation with either 5 μl (20 μg) anti-MUC1 antibody (B27.29) or 20 μl of anti-galectin-3 antibody for 2 h at 4 °C followed by the addition of 50 μl of protein A-agarose for a further hour. After washing, the immunoprecipitates were retrieved by mixing the beads with 40 μl of SDS sample buffer and boiling for 10 min before separation on either a 4% (for MUC1 analysis) or 12% (for galectin-3 analysis) SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose membranes and probed with either anti-MUC1 or anti-galectin-3 primary antibodies. After application of the peroxidase-conjugated secondary antibodies, the blots were developed with SuperSignal West Dura Extended Duration Substrate (Pierce) and visualized using a Fluor-S Imager (Bio-Rad).
Desialylation and Deglycosylation—MUC1 immunoprecipitates prepared as described above were divided into six equal aliquots and incubated with or without 0.02 unit/ml N-glycanase, 0.02 unit/ml A. ureafaciens sialidase, which cleaves 2-3, 2-6, and 2-8-linked sialic acid, 0.02 unit/ml S. pneumoniae O-glycanase, which is highly specific for unsubstituted O-linked Galβ1,3GalNAcα−, or 0.02 unit/ml sialidase plus 0.02 unit/ml O-glycanase for 16 h at 37 °C (37, 38). The immunoprecipitates were separated on SDS-PAGE (4% running gel and 3.75% stack gel), transferred to nitrocellulose membranes, and probed with 1 μg/ml recombinant galectin-3 and then anti-galectin-3 antibody followed by peroxidase-conjugated secondary antibody.

In some experiments, 30 μg of cell lysates of MUC1-transfected HCA1.7+/− or MDE9.2+/− cells were separated on SDS-PAGE and transferred to nitrocellulose membranes. The membranes were then incubated with A. ureafaciens sialidase (0.02 unit/ml) for 16 h at 37 °C before probing with anti-TF5 antibody.

In other experiments, subconfluent MDE9.2+, MDE9.2−, HCA1.7+, or HCA1.7− cells were incubated with or without A. ureafaciens sialidase (0.02 unit/ml), or O-glycanase (0.02 unit/ml) for 1–3 h at 37 °C before lysis of the cells and followed by blotting with anti-MUC1, anti-TF5 antibody, or TF-binding peanut lectin (PNA).

Cell Adhesion to HUVEC—Subconfluent epithelial cancer (ZR-75-1, HT29-5F7, or HT29) cells or MUC1 transiently transfected HCA1.7+/− or MDE9.2+/− cells cultured in 24-well plates were washed with PBS and labeled with 5 μg/ml Dil fluorescent cell labeling solution in serum-free Dulbecco’s modified Eagle’s medium for 30 min at 37 °C. The cells were washed with PBS and treated with a nonenzymatic cell dissociation solution (Sigma) that releases the cells from the culture plates while keeping the cell membrane proteins intact. After washing, 5 × 10^4 cells were incubated with or without recombinant galectin-3 in the presence or absence of 50 mM lactose for 30 min at 37 °C before application for 1 h at 37 °C to a HUVEC monolayer cultured on chamber slides. The chamber slides were then gently washed with PBS and inverted for 10 min at room temperature. The slides were mounted, and the fluorescent-labeled cells were counted between three and ten randomly chosen low power fields using an Olympus B51 fluorescent microscope with a 20× objective (200× magnifications).

Effect of Antibodies against Adhesion Molecules on Cell Adhesion to HUVEC—A range of monoclonal antibodies against potentially relevant adhesion molecules (E-Selectin, CD44H, Integrin-β1, ICAM-1, and VCAM-1) was incubated with HUVEC cells at 25 μg/ml for 30 min at 37 °C and remained present during the subsequent 1-h cell adhesion assay as described above.

Effect of Galectin-3 on MUC1 Cell Surface Localization—Subconfluent HCA1.7+/− cells were released from the culture plates using the nonenzymatic cell dissociation solution. After washing, 10^4 cells were incubated with or without 0.5–1 μg/ml recombinant galectin-3 for 1 h at 37 °C. The cell suspensions were then applied to polylysine-coated slides for 30 min at room temperature. After gentle washing, the cells were fixed with 2% paraformaldehyde, blocked with 5% normal goat serum/PBS, and probed with anti-MUC1 antibody, followed by fluorescent-labeled secondary antibody. MUC1 localization was visualized using an Olympus B51 fluorescent microscope. Focal rearrangement of cell surface MUC1 expression was scored by two observers blinded to the cell treatment who counted the percentage of cells lacking a continuous rim of MUC1 in eight randomly selected low power fields.

Laser Scanning Confocal Microscopy of Cell Adhesion to HUVEC—Samples of epithelial cells adherent to HUVEC were prepared as described above. Before introduction of recombinant galectin-3-treated HCA1.7+/− cells to the HUVEC monolayer, the HUVEC cells were prelabeled with Dil (5 μl/well) cell labeling solution for 30 min at 37 °C. After interaction of the HCA1.7+/− HUVEC cells at 37 °C for 30 min, the cells were washed with PBS, fixed in 2% paraformaldehyde/PBS for 20 min, treated with 0.1% Triton X-100 for 5 min, and then treated with 5% normal goat serum/PBS for 30 min. B27.29 anti-MUC1 antibody at 0.5 μg/ml in 1% bovine serum albumin/PBS was introduced followed by fluorescein isothiocyanate-labeled secondary antibody. The laser scanning confocal microscopy was performed with Leica SP2 laser scanning confocal microscope.
MUC1 immunoprecipitates were pretreated with N-glycanase, but binding was markedly reduced if the immunoprecipitates were pretreated with *Streptococcus* endo-
N-acetylglactosaminidase (*O*-gly-
canase), which is highly specific for liberating unsubstituted TF from serine or threonine residues (38). Pretreatment of MUC1 immunoprecipitates with *A. ureafaciens* sialidase, which cleaves nonreduc-
ting terminal α2–3, 2–6, and 2–8-
linked sialic acid from galactose, 
N-acetylgalactosamine and N ace-
tyglycosamine residues, showed the characteristic reduced mobility in SDS-PAGE that results from the removal of the negatively charged sialic acids (31). Desialylation of MUC1 greatly enhanced galectin-3 binding to MUC1, which was then markedly reduced following additional *O*-glycanase treatment. To-
gether, these results suggest that galectin-3 interacts directly with 

MUC1 and that this interaction is mediated, to a large extent, by binding of galectin-3 to the unsubstituted TF disaccharide on MUC1.

**Recombinant Galectin-3 Enhances Epithelial Cancer Cell Adhesion to HUVEC**—We next investigated the functional sig-
nificance of the interaction of galectin-3 with MUC1. It had been reported previously that endothelial cell–associated galec-
tin-3 mediates heterotypic adhesion of cancer cells to endothe-
lia via binding to cancer-associated TF antigen expressed by unknown cell surface molecules (10–14). This together with the presence of increased circulating galectin-3 concentrations in cancer patients and the overexpression in cancer of MUC1 bearing increased copy numbers of unsubstituted TF, prompted us to investigate the role of the interaction between galectin-3 and cancer-associated MUC1 in cancer cell adhesion to endothelium.

We therefore preincubated MUC1-expressing epithelial cancer cells with recombinant galectin-3 at various concentra-
tions and subsequently tested the adhesion of the cells to 
HUVECs. It was found that galectin-3 at concentrations (0.2–
1.0 µg/ml) similar to those found in patients with metastatic
breast or colon cancer induces a significant increase of cancer cell adhesion of human breast cancer cells to the HUVEC monolayer. At 1 µg/ml, recombinant galectin-3 increased adhesion of ZR-75-1 human breast cancer cells to HUVEC by 111% (111 ± 21%, mean ± S.D., *p* < 0.001, ANOVA) (Fig. 2). At similar concentration, recombinant galectin-3 caused little change of the adhesion of parental (standard) HT29 cells (data not shown) but 93% (93 ± 17%, *p* < 0.001, ANOVA) increased adhesion of HT29-5F7, a subpopulation of HT29 cells that have greater MUC1 expression than the parental HT29 cells (35, 39). In the absence of galectin-3, an average of 35 (35 ± 5) ZR-75-1
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FIGURE 3. Galectin-3 has differing effects on adhesion of MUC1-positive transfectants and MUC1-negative revertants to HUVEC. A, HCA1.7+/−- and MED9.2+/−- cell lysates (30 μg) were immunoblotted with anti-MUC1 antibody (B27.29) showing high MUC1 expression in MUC1 transfectants HCA1.7+ and MED9.2+ but not in their revertants HCA1.7− and MED9.2−. Incubation of the cells with 1 μg/ml recombinant galectin-3 caused increased cell adhesion to HUVEC of MUC1 transfectants HCA1.7+ but not of revertant HCA1.7− nor of either transfected MED9.2+ or revertant MED9.2−. The data in B and C are shown as the means ± S.D. from five (B) and three (C) separate experiments. *, p < 0.01, unpaired t test.

Recombinant Galectin-3 Selectively Enhances Adhesion of MUC1-Transfected Cells to HUVEC—To determine whether the observed effects of recombinant galectin-3 on cancer cell adhesion to HUVEC were related to interaction with MUC1, we assessed the effect of recombinant galectin-3 on cell adhesion in MUC1-transfected, MUC1-positive, and in MUC1-negative (revertant) cells that had lost MUC1 expression. Normal human breast epithelial HBL-100 cells and Ras-transformed Madin-Darby canine kidney epithelial-like MDCK-Ras-e cells, both naturally expressing no MUC1 (26), were transfected with MUC1, and the effects of recombinant galectin-3 on MUC1-positive (transfectant HCA1.7+ and MED9.2+) and -negative (revertant HCA1.7− and MED9.2−) cells were determined. The MUC1 transfectants HCA1.7+ and MED9.2+ showed high levels of MUC1 expression, comparable with those found on breast cancer ZR-75-1 cells (26), and the MUC1-negative revertants HCA1.7− and MED9.2− showed no detectable MUC1 when assessed by MUC1 immunoblotting (Fig. 3A).

It was found that recombinant galectin-3 at 0.5−1 μg/ml increased adhesion of MUC1 transfectant HCA1.7+ cells to HUVEC. At 1 μg/ml, recombinant galectin-3 caused 112% (112 ± 13%, p < 0.01) increased adhesion of HCA1.7+, but not of MUC1-negative revertant, HCA1.7−, cells (5 ± 19%) (Fig. 3B). In the absence of galectin-3, an average of 2.5 (2.5 ± 1.4) HCA1.7+ cells and 5.8 (5.8 ± 3.0) HCA1.7− cells were adherent per randomly selected low power field.

Unexpectedly, we found that, although the MUC1-transfected MED9.2+ cells express a similarly high amount of MUC1 to that expressed by HCA1.7+ and ZR-75-1 cells, recombinant galectin-3 (0.5−1 μg/ml) had no significant effect on the adhesion of these cells to HUVEC (Fig. 3C). This prompted us to analyze the glycosylation of the MUC1 expressed by these two MUC1-transfected cell lines. It was found that the MUC1 expressed by HCA1.7+ cells showed strong affinity for the TF-binding peanut lectin (PNA) and for anti-TF antibody (anti-TF5), whereas the MUC1 expressed by MED9.2+ cells was not bound by either PNA or anti-TF5 antibody when assessed by lectin/immunoblotting using the anti-TF5 antibody. MUC1 in MED9.2+ cells shows weak binding to the TF and sialyl-TF-binding lectins ABL and jacalin, but only MUC1 in MED9.2+ cells shows weak binding by MAL-II. Sialidase pretreatment of the proteins preblotted on nitrocellulose membrane allows MUC1 in MED9.2+ to be strongly bound by anti-TF5. No apparent expression of sialyl-Tn and Tn was seen on MUC1 in either HCA1.7+ or MED9.2+ cells (h and i, respectively). MW, molecular mass.

FIGURE 4. Identification of TF expression by MUC1 in HCA1.7+ cells and sialyl-TF by MUC1 in MED9.2+ transfectants. Immunoblotting and lectin blotting of MED9.2+ (lanes 1) or HCA1.7+ cell lysates (30 μg) (lanes 2) was performed using anti-MUC1 (a), HRP-PNA (b), anti-TF5 (c), HRPAVL (d), biotin-JAC (jacalin) (e), biotin-MAL-II (f), anti-STn antibody (h), and biotin-GSL (i). In g, the blot was pretreated with A. ureafaciens sialidase for 16 h before blotting with anti-TF5 antibody. MUC1 in HCA1.7+ cells, but not in MED9.2+, shows strong binding by TF-binding PNA and anti-TF5. MUC1 in both HCA1.7+ and MED9.2+ cells shows binding by the TF and sialyl-TF-binding lectins ABL and jacalin, but only MUC1 in MED9.2+ cells shows weak binding by MAL-II. Sialidase pretreatment of the proteins preblotted on nitrocellulose membrane allows MUC1 in MED9.2+ to be strongly bound by anti-TF5. No apparent expression of sialyl-Tn and Tn was seen on MUC1 in either HCA1.7+ or MED9.2+ cells (h and i, respectively). MW, molecular mass.

MUC1 to that expressed by HCA1.7+ and ZR-75-1 cells, recombinant galectin-3 (0.5−1 μg/ml) had no significant effect on the adhesion of these cells to HUVEC (Fig. 3C). This prompted us to analyze the glycosylation of the MUC1 expressed by these two MUC1-transfected cell lines. It was found that the MUC1 expressed by HCA1.7+ cells showed strong affinity for the TF-binding peanut lectin (PNA) and for anti-TF antibody (anti-TF5), whereas the MUC1 expressed by MED9.2+ cells was not bound by either PNA or anti-TF5 antibody when assessed by lectin/immunoblotting (Fig. 4, a–c). However, the MUC1 in HCA1.7+ as well as that in MED9.2+ cells was strongly bound by the lectins Agaricus bisporus (ABL) and jacalin (Artocarpus integripilosa) that bind sialylated TF as well as TF (Fig. 4, d and e). This suggested that MUC1 in HCA1.7+ cells expresses unsubstituted TF, whereas the MUC1 in MED9.2+ cells expresses sialylated TF. This was supported by lectin blotting using the sialic acid-binding M. amurenensis lectin (MAL-II). MAL-II showed weak but definite binding to MUC1 only in MED9.2+ but not in HCA1.7+ cells (Fig. 4f). Furthermore, when cell lysates blotted on nitrocellulose membrane were treated first with A. ureafaciens sialidase then with lectins, Agaricus bisporus (ABL) and jacalin (Artocarpus integripilosa) that bind sialylated TF, but not in MED9.2+ cells was not found to carry either Tn (N-acetylgalactosamine α−) or sialylated Tn antigens when assessed by lectin/immunoblotting using the N-acetylgalactosamine-binding lectin from G. simplificofolia (GSL) or anti-sialyl-Tn antibody (B195.3) (Fig. 4, h and i). Together, these results suggest that the MUC1 molecules in HCA1.7+...
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Galectin-3 Alters MUC1 Cell Surface Localization—To gain insight into the mechanism of promotion of cell adhesion to endothelium by galectin-3-MUC1 interaction, we next assessed the effect of recombinant galectin-3 on MUC1 cell surface localization. It was found that MUC1 spreads almost homogenously around the whole cell surface of untreated HCA1.7+ cells (Fig. 6a). After treatment of the cells with 1 μg/ml recombinant galectin-3 for 30 min, MUC1 lost its homogenous cell surface localization in suspension (Fig. 6b). When scored in two low power fields for each treatment by an observer blinded as to the cell treatments, focal loss of circumferential MUC1 staining was seen in 65% (44 of 68 cells) of the galectin-3-treated cells compared with 7% (4 of 58 cells) of the untreated cells ($p < 0.0001$ by Fisher’s exact test; Fig. 6). Confocal microscopy of HCA1.7+ cells adhered to the HUVEC monolayer with immunostaining for MUC1 shows that MUC1 is absent at the epithelial-endothelial contacts (Fig. 6C).

Co-incubation with Anti-E-selectin or Anti-CD44H Antibody Reduces Galectin-3-MUC1-mediated Cell Adhesion to HUVEC—To gain insight into the identity of the adhesion molecules in galectin3-MUC1-mediated cell adhesion, we determined the effect of co-incubation with antibodies against endothelial-associated adhesion molecules. HUVEC are known to express various cell adhesion molecules including ICAM-1, VCAM-1, integrins, CD44H, and E-Selectin (40, 41). It was found that pretreatment of the HUVEC monolayer with 25 μg/ml antibodies against E-Selectin or CD44H before introduction of galectin-3-treated HCA1.7+ cells resulted in significant reduction (82 ± 14%, $p < 0.001$ and 72 ± 13%, $p < 0.005$, respectively, ANOVA) of the cell to recombinant galectin-3 are largely due to differences in glycosylation of MUC1, with MDE9.2+ expressing sialyl-TF and HCA1.7+ expressing unsubstituted TF.

To further investigate the role of TF structure on MUC1 in galectin-3-mediated cell adhesion, we pretreated live HCA1.7+/− cells with O-glycanase for 2 h at 37 °C. This resulted in a 35% reduction of TF expression on MUC1 in HCA1.7+ cells when assessed by PNA blotting (Fig. 5C). O-Glycanase treatment of the cells resulted in a 46% decrease in the galectin-3 (1 μg/ml)-induced adhesion of HCA1.7+ to HUVEC but did not affect the adhesion of HCA1.7− cells (Fig. 5D). This provides further evidence that galectin-3 binding to MUC1 requires the presence of unsubstituted TF.

FIGURE 5. Effect of sialidase or O-glycanase treatment of MUC1 transfectants/revertant cells on TF/MUC1 expression and on galectin-3-mediated cell adhesion. A, MDE9.2− cells were treated with A. ureafaciens sialidase (0.02 unit/ml) for 0, 1, or 3 h at 37 °C before lysis and blotting with anti-MUC1 (B27.29) (left panel) or anti-TF (TF5) antibody (right panel). Sialidase treatment of the cells results in exposure of TF antigen on MUC1 in MDE9.2− cells and a reduced MUC1 mobility on SDS-electrophoresis. B, MDE9.2+/− cells were treated with A. ureafaciens sialidase for 1 h (0.02 unit/ml) followed by incubation with 1 μg/ml recombinant galectin-3 before application to a HUVEC monolayer. Sialidase pretreatment of the cells results in increased cell adhesion to HUVEC of MDE9.2+/− but not MDE9.2− cells. The data in B are shown as the means ± S.D. from two experiments each performed in triplicate. C, HCA1.7− cells were treated with or without O-glycanase (0.02 unit/ml) for 2 h at 37 °C before lysis and blotting with anti-MUC1 (B27.29) antibody (left panel) or TF-binding PNA (right panel) (two gels). O-Glycanase treatment of the cells results in reduction of TF expression on MUC1. D, HCA1.7+/− cells were treated with or without O-glycanase (0.02 unit/ml) for 2 h followed by incubation with or without 1 μg/ml recombinant galectin-3 before application to a HUVEC monolayer. O-Glycanase pretreatment of the cells results in reduction of cell adhesion to HUVEC of HCA1.7+/− but not HCA1.7− cells. The data in B and D are shown as the means ± S.D. from three separate experiments. *, $p < 0.01$; **, $p < 0.001$, unpaired t test. MW, molecular mass.

cells carry predominantly the unsubstituted TF structure, whereas the MUC1 in MDE9.2+ cells carries predominantly the sialylated TF structure. This is in keeping with the finding that the binding of recombinant galectin-3 to MUC1 is largely mediated through binding of galectin-3 to unsubstituted TF carried on MUC1 (Fig. 1).

Effect of Sialidase Treatment on Galectin-3-mediated Adhesion to HUVEC—Because the experiments above suggested that the different responses of HCA1.7+ and MDE9.2+ cells to galectin-3 in cell adhesion might be accounted for by differences in sialylation of TF carried by MUC1, we pretreated live MDE9.2+ cells with A. ureafaciens sialidase for 1–3 h at 37 °C. This treatment removes the terminal sialic acid residues from MUC1 and allowed strong binding of the MUC1 by anti-TF antibody (Fig. 5A). The MUC1 molecule from the lysed cells again showed the characteristic reduced mobility in SDS-PAGE after sialidase treatment (Fig. 5A).

Sialidase pretreatment of the MDE9.2+ cells, followed by galectin-3 incubation resulted in 102% (102 ± 22%, $p < 0.01$) increased adhesion to HUVEC but did not affect significantly the adhesion of MDE9.2− cells (9 ± 18%, $p = 0.4$) (Fig. 5B). Sialidase pretreatment of HCA1.7+ cells did not affect the cell adhesion induced by recombinant galectin-3 (data not shown). These results strongly suggest that the different adhesion responses of the MUC1 transfectants HCA1.7+ and MDE9.2+ to recombinant galectin-3 are largely due to differences in glycosylation of MUC1, with MDE9.2+ expressing sialyl-TF and HCA1.7+ expressing unsubstituted TF.
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FIGURE 6. Galectin-3 induces changes in MUC1 cell surface localization. HCA1.7+ cells were released from culture using a nonenzymatic cell dissociation solution and treated without (a) or with (b) 1 µg/ml recombinant galectin-3 for 30 min before fixation, probing with anti-MUC1 antibody/fluorescein isothiocyanate-conjugated secondary antibody, and viewing by fluorescent microscopy after blind labeling. 44 of 68 (65%) of the cells treated with recombinant galectin-3 (1 µg/ml) showed a loss of continuous circumferential staining for MUC1 compared with only 4 of 58 (7%) untreated cells (p < 0.0001 by Fisher’s exact test). In C, HCA1.7+ cells were treated with 1 µg/ml recombinant galectin-3 for 30 min before their introduction to Dil-prelabeled HUVEC monolayer (red color). After fixation, permeabilization and probing with anti-MUC1 antibody/fluorescein isothiocyanate-conjugated secondary antibody, laser scanning confocal microscopy was performed, and the z-stacks were analyzed in orthogonal projections (x–z sections). MUC1 is shown to be absent at the heterotypic intercellular contacts with polarization away from the contact point (yellow arrow). Scale bar, 10 µm.

FIGURE 7. Pretreatment of HUVEC with anti-E-selectin or anti-CD44H antibody reduces galectin-3-MUC1-mediated cell adhesion. HUVEC monolayers were separately pretreated with or without 25 µg/ml of each antibody before the introduction of recombinant galectin-3-treated or untreated HCA1.7+ cells. Pretreatment with antibodies against E-Selectin or CD44H, but not integrin-β1, VCAM-1, or ICAM1, resulted in significant reduction of the cell adhesion to HUVEC. The data are shown as the means ± S.E. from three separate experiments, *, p < 0.005; **, p < 0.001; NS, not significant, when compared with galectin-3-treated cells (ANOVA).

FIGURE 8. Proposed action of galectin-3-MUC1 interaction. Cancer-associated, TF-expressing, MUC1 expressed on the cancer cell surface shields the smaller cell adhesion molecules (or ligands to adhesion molecules). Binding of recombinant or circulating galectin-3 to TF on MUC1 causes redistribution of MUC1 on the cell surface leading to exposure of the smaller cell adhesion molecules (or ligands to adhesion molecules), thus allowing epithelial-endothelial interaction via ligands such as E-Selectin and CD44H.

adhesion (Fig. 7). Pretreatment of the HUVEC monolayer with antibodies against ICAM-1 resulted in a small but insignificant reduction (35 ± 22%, p = 0.07, ANOVA), and pretreatment of HUVEC with antibodies against integrin-β1 and VCAM-1 did not affect galectin-3-induced cell adhesion.

DISCUSSION

These studies show that cancer-associated MUC1 is a natural ligand for galectin-3 and that the MUC1-galectin-3 interaction is mediated largely via binding of galectin-3 to the TF oncofetal carbohydrate antigen on MUC1. Recombinant galectin-3 at concentrations similar to those found in the sera of patients with metastatic cancer causes marked increase of adhesion of breast and colon cancer cells to human umbilical vein endothelial cells. This effect is mediated largely by binding of galectin-3 to MUC1 via its interaction with unsubstituted TF. Although previous studies have shown an association between cell surface and intracellular galectin-3 and cancer development (1), the functional significance of elevated circulating galectin-3 in cancer patients was unknown. The present study suggests for the first time that circulating galectin-3, by interaction with cancer-associated MUC1, may play a critical role in cancer cell adhesion to endothelium and hence in cancer progression and metastasis.

The observation that incubation of the MUC1-transfected HCA1.7+ cells with recombinant galectin-3 alters MUC1 cell surface localization suggests that this process may be at least partly responsible for the increased cell adhesion as a consequence of revealing adhesion molecules (or ligands) that would otherwise be concealed by the large MUC1 molecule. This leads us to propose a working model of galectin-3-MUC1 interaction (Fig. 8) in which cancer-associated MUC1 on the surface of cancer cells shields the smaller cell adhesion molecules (or ligands to adhesion molecules) and prohibits cancer cell interaction with adjacent cells. Binding of recombinant or circulating galectin-3 to unsialylated TF on cancer-associated MUC1, both of which, i.e. unsialylated TF and MUC1, are overexpressed in cancer cells, causes redistribution of MUC1 on the cell surface and the exposure of the smaller cell adhesion molecules (or ligands to adhesion molecules), thus allowing interaction between the cancer cells and the endothelium.

This model is supported by evidence that MUC1 polarization in human breast cancer cells induced by an anti-MUC1 antibody exposes cell adhesion molecules including integrins and cause adherence of these cells to extracellular matrix proteins in vitro (26). The inhibitory effect of E-Selectin and CD44H antibodies on galectin-3-induced HCA1.7+ adhesion to HUVEC further supports this model. E-Selectin is an adhesion molecule that has a key role in mediating lymphocyte-endothelial and cancer-endothelial interactions (42, 43). CD44H is a transmembrane glycoprotein expressed by many cell types including endothelial (44) and epithelial (40) cells. It is also a known ligand for E-Selectin (45) and has previously been shown to be involved in mediating melanoma cell adhesion to HUVEC (46).

Our model and the experimental data supporting it imply a multivalent action of circulating galectin-3 for it to exert its effect on cancer cell adhesion to endothelium. Although galec-
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Galin-3 exists as a single polypeptide protein with one carbohydrate recognition domain, it is known to oligomerize at higher concentrations (8, 47, 48) or when it binds to multivalent carbohydrates (49) and then to function in a multivalent fashion. Indeed, galactin-3 homodimers have been found in the sera of cancer patients (2).

Our results support a dual adhesive and anti-adhesive function for cancer-associated MUC1. It is known that cancer-associated MUC1 can function as an adhesion molecule when its overexpression on the cell surface reduces interaction of cells with their neighbor cells and thus helps the detachment and invasion of cancer cells through the basement membrane (26, 30–32). Conversely, MUC1 can behave as an adhesion molecule when in contact with lectin-like molecules such as ICAM-1 on B cells (50) and thus enhance cell aggregation. It is likely that both the adhesive and anti-adhesive properties of MUC1 are important at different stages of the development and progression of cancer.

Although it has long been known that altered cell surface glycosylation is common in malignant and premalignant epithelia (51, 52), it is only recently that the functional significance of these glycosylation changes has begun to be demonstrated. There is increasing evidence that these glycosylation changes may affect cancer cell adhesion, mobility, and invasion (53, 54). The demonstration that the expression of the unsubstituted TF disaccharide by cell surface MUC1 allows galectin-3 to induce cancer cell adhesion to endothelial cells suggests that the increased expression of unsialylated TF by cancer cells, one of the commonest glycosylation changes in cancer (15, 55), may directly encourage cancer metastasis.

This study not only implies a critical role for circulating galectin-3 in cancer metastasis but also highlights the functional importance of altered cell surface glycosylation in the development and progression of cancer.

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