Colon Cancer Mucin: A New Ligand for the β-Galactoside-binding Protein Galectin-3

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Abstract

Galectin-3, an endogenous β-galactoside-binding lectin, is present on colon cancer cells and may play a role in metastasis. Galectin-3 binds poly-N-acetyllactosamine structures on glycoproteins, but its natural ligands remain to be fully defined. Galectin-3 bound to purified native and desialylated colon cancer mucin in a concentration-dependent manner, which was completely inhibited by 0.1 m lactose, the competitive inhibitory sugar for this protein. Mucin purified from highly metastatic LS-Lim6 human colon cancer cells bound galectin-3 to a 2-fold greater extent than mucin from low-metastatic parental cell line LS174T. Desialylation increased binding to mucin >4-fold. Mucin purified from LS-B colon cancer cells is fully glycosylated and bound >40-fold more galectin-3 than mucin purified from clonal cell line LS-C, which produces mucin lacking peripheral carbohydrate structures. Endogenous galectin-3 was detected by Western analysis in all cell lines, and its expression was related to mucin production and metastatic capacity. When serum from a patient with metastatic colorectal cancer was chromatographed on Superose 6, >70% of galectin-3 ligand was identified as circulating mucin. Colon cancer mucin is a newly identified ligand for galectin-3, and binding of galectin-3 to mucins depends on peripheral carbohydrate structures. Binding of this endogenous lectin to mucins may influence cellular interactions that play a role in colon cancer metastasis.

Introduction

Endogenous β-galactoside-binding lectins constitute a gene family of widely distributed carbohydrate-binding proteins that have been implicated in cell growth, differentiation, adhesion, transformation, and metastasis through interactions with specific ligands (1). The endogenous human carbohydrate-binding protein galectin-3 (also known as Mac-2, CBP-35, IgEBP, CBP-30, RL-29, L-29, H-31, and LBL) is one such lectin. Although the structure of galectin-3 has been well established (2, 3), its precise biological role remains to be determined. Galectin-3 is found at elevated levels in a variety of neoplastic cells, and recent evidence suggests that it is involved in tumor metastasis in vivo (4–6). We have previously demonstrated that galectin-3 expression correlates with tumor progression in the colon (7), including the ability of colon cancer cells to metastasize. Furthermore, enhanced galectin-3 expression correlates with decreased patient survival. Galectin-3 acts as a receptor for ligands containing poly-N-acetyllactosamine (Galβ4GlcNAcβ3Galβ4GlcNAc) sequences, but little is known about its natural ligands in different cells. Several potential ligands for galectin-3, including lysosomal-associated membrane proteins 1 and 2, IgE, laminin, M2BP-1, M2BP-2, and Mac-2-BP, have been described (8, 9). Because mucin glycoproteins are major products of colon cancer cells, and mucin production has been correlated with their metastatic capacity (10, 11), we sought to determine whether colon cancer mucin serves as a ligand for galectin-3.

Materials and Methods

Materials. Peroxidase-labeled lectins and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Rat monoclonal anti-galectin-3 was obtained from culture supernatants of hybridoma TIB-166 (American Type Culture Collection). Sepharose CL-4B and Superose 6 were purchased from Pharmacia Fine Chemicals (Piscataway NJ).

Cell Lines. Parental cell line LS174T was derived from a well-differentiated colorectal adenocarcinoma and has been characterized extensively (10–12). The selection and initial characterization of the LS-Lim6, LS-B, and LS-C colon cancer cell lines have been described previously (12, 13). LS-Lim6 is a derivative of LS174T with high liver-metastasizing ability during cecal growth and high liver-colonizing capacity after splenic-portal injection in athymic mice (10, 11). LS-C and LS-B are clonal derivatives of LS174T that differ in the extent of glycosylation of their mucin carbohydrate chains. LS-C is a high-sialyl Tf-expressing variant that produces truncated mucin oligosaccharides, whereas LS-B produces more fully glycosylated mucin (14). HM7 is a high mucin-producing variant of LS174T (10, 11).

Purification and Analysis of Secreted Mucins. Cells were seeded at low density into tissue culture flasks in DMEM with 10% FCS and incubated overnight to allow attachment. The medium was changed to serum-free Opti-MEM (Life Technologies, Inc., Grand Island, NY), and cells were grown for an additional 6 days with two medium changes. The medium was pooled, dialyzed against water, and lyophilized. Concentrated medium was chromatographed on a column (2.5 X 115 cm) of Sepharose CL-4B in 10 mM Tris (pH 8). The void volume was pooled, dialyzed, lyophilized, and subjected to Sephadex G-50 (Pharmacia) column chromatography. Neutral sugars were assayed with the phenol-sulfuric acid assay, using glucose as the standard (15). Sialic acids were assayed using a high pressure liquid chromatographic adaptation of the thiobarbituric acid assay (16), with NeuAc as the standard. Neutral sugars were assayed with the phenol-sulfuric acid assay, using glucose as the standard (15). Sialic acids were assayed using a high pressure liquid chromatographic adaptation of the thiobarbituric acid assay (16), with NeuAc as the standard. Binding of peroxidase-labeled PNA, peroxidase-labeled VVA-B4, and peroxidase-labeled RCA was assayed as described previously (14, 17).

Human Recombinant Galectin-3 Expression and Purification. Recombinant galectin-3 was expressed in Escherichia coli and purified by affinity chromatography as described previously in detail (2).

Galectin-3 Binding to Mucins. Purified mucins were adsorbed to microtiter plates overnight, blocked with 1% BSA/PBS, and incubated sequentially with purified galectin-3 (100–250 ng/well), monoclonal anti-galectin-3, biotinylated rabbit anti-galectin-3, and avidin-biotin-complexed peroxidase. Between steps, the plates were washed three times with PBS. Peroxidase activity was detected with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) reagent, reading absorbance at 405 nm. Controls containing 0.1 m lactose were included as blanks. Data are the result of at least three determinations for each condition.

Immunoblot Analysis. Colon cancer cells were harvested by scraping in PBS and homogenized by sonication. Aliquots of equal homogenate protein were analyzed by immunoblotting using antibodies specific for mucin carbohydrate epitopes.

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1 The abbreviations used are: PNA, peanut agglutinin; VVA, Vicia villosa agglutinin; VVA-B4, Vicia villosa agglutinin; Sl, sialic acid; RCA, Ricinus communis agglutinin; CEA, carcinoembryonic antigen.
were resolved by 10% SDS-PAGE and transferred to an Immobilon P filter. The filter was incubated with rat anti-galectin-3 and sheep antirat IgG as described (18). Autoradiographs were analyzed with the use of a Bio-Rad GS-70 imaging densitometer with Molecular Analyst software (Bio-Rad Laboratories, Hercules, CA).

Characterization of Galectin-3 Ligands in the Serum of a Patient with Metastatic Colon Cancer. Serum was obtained from a patient with moderately differentiated adenocarcinoma of the transverse colon with extensive metastasis to the liver documented at laparotomy and reflected by a CEA level of 30,000 ng/ml. Serum was chromatographed on a column of Superose 6 (1.0 × 30.6 cm) eluted with 50 mM NH$_4$HCO$_3$ at a flow rate of 0.5 ml/min. Fractions of 1 ml were collected. Aliquots were adsorbed to polystyrene microtiter plates and assayed for binding of galectin-3, antibodies, and lectins. Endogenous galectin-3 was assayed by ELISA with no recombinant galectin-3 added. A sample of normal human serum was analyzed in the same fashion. In addition, aliquots eluted from the column were subjected to immunoblot analysis using rat anti-galectin-3.

Results

Binding of Galectin-3 to Purified Mucins. Mucin may be detected in the serum and is the major glycoprotein produced by colon cancer cells. Because galectin-3 is also expressed by these cells (7), we sought to determine whether colon cancer mucin and galectin-3 have a receptor-ligand relationship. Mucin was purified from spent medium of human colon LS-Lim6 and LS174T cells. Galectin-3 binding to high-M$_r$ glycoproteins at the void volume of Sepharose CL-4B was 5-fold greater than binding to low-M$_r$ glycoproteins secreted by metastatic LS-Lim6 colon cancer cells. The overall yield of mucin protein secreted by Lim6 cells was 2-fold higher than that secreted by LS174T cells (yield, 0.49 versus 0.23 µg/ml), and mucin purified from highly metastatic Lim6 cells had a higher content of carbohydrate (2.2 mg hexose and 1.1 mg sialic acid/mg protein) than mucin from low-metastatic counterpart LS174T cells (1.8 mg hexose and 0.6 mg sialic acid/mg protein).

Mucin purified from Lim6 cells bound galectin-3 in a dose-dependent manner and to a 2-fold greater extent than mucin from low-metastatic cell line LS174T (Fig. 1A). For both Lim6 and LS174T mucins, galectin-3 binding was inhibited by 0.1 M lactose. Desialylation of LS-Lim6 mucin increased galectin-3 binding >4-fold (Fig. 1B). Similar results were obtained with mucin purified from LS174T cells and from nude mouse xenografts of HM-7 colon cancer cells (not shown). This indicates that much of the recognition domain for galectin-3 in colon cancer mucins is probably cryptic due to sialylation, in keeping with the high sialic acid content of these mucins.

To further characterize the binding of galectin-3 to the carbohydrate side chains of mucin glycoproteins, we next purified mucins from serum-free medium of human colon cancer cell lines that vary in peripheral and core carbohydrate (12, 19, 20). LS-B cells produce fully glycosylated mucin, whereas LS-C cells produce mucin enriched in core carbohydrate antigens. LS-B mucin has a higher carbohydrate content (1.46 mg hexose and 0.74 mg sialic acid/mg protein) compared with LS-C mucin (0.25 mg hexose and 0.47 mg sialic acid/mg protein). Native mucin purified from LS-B cells bound at least 40-fold more galectin-3 than LS-C mucin (Fig. 2). This suggests that peripheral carbohydrates are the targets for galectin-3 binding. Desialylation of LS-B mucin greatly increased the binding of galectin-3, whereas little binding of galectin-3 to LS-C mucin was observed even after desialylation (not shown).

Endogenous Galectin-3 in Colon Cancer Cells. Previous results indicate that the expression of galectin-3 is higher in colon cancers than in normal colon, and that metastases have higher levels of galectin-3 than paired primary tumors (7). To determine whether the content of endogenous galectin-3 correlates with the metastatic phenotype, protein extracts of human colon carcinoma cells were examined by immunoblotting using anti-galectin-3. Metastatic LS-Lim6 cells expressed 5.6-fold more galectin-3 than parental LS174T, whereas LS-B and LS-C cells expressed similar amounts of lectin (not shown).

Gel Filtration Analysis of Galectin-3 Ligand in Serum. Serum from a patient with metastatic colon cancer was subjected to size exclusion chromatography on a column of Superose 6 and analyzed for binding of lectins and galectin-3 (Fig. 3). Galectin-3 ligands were detected by assaying binding of exogenous galectin-3 to polystyrene-immobilized column fractions. Binding of galectin-3 was predominately at the mucin-containing void volume of Superose 6 (Fig. 3A). The amount of endogenous galectin-3 in the void volume fraction (blank subtracted in Fig. 3A) corresponded to 6% of the exogenous galectin-3 bound. Endogenous galectin-3 was also detected in the void volume fraction on immunoblot analysis of the column fractions. When normal human serum was analyzed in the same fashion, the
Galectin-3 expression has been recently correlated with tumor progression in the colon (7). Metastatic foci also express more galectin-3 than the primary tumors from which they were derived. Galectin-3 ligands that have been previously associated with colon cancer cells include M2BP-1 (98 kDa) and M2BP-2 (70 kDa; Ref. 23), laminin (24, 25), CEA (26), and lysosome-associated membrane glycoproteins (26).

Because mucins are highly glycosylated and contain polylactosamine structures, we examined the ability of colon cancer mucins to act as ligands for galectin-3. Mucins were purified from a variety of human colon cancer cells that differ in metastatic potential or in their expression of mucin-associated carbohydrate antigens. Galectin-3 bound to purified secreted mucins in a concentration-dependent manner, the specificity of which was indicated by complete abrogation of the binding by 0.1 m lactose. Mucin from LS-B cells is fully glycosylated, whereas clonal cell line LS-C produces mucin with truncated glycoproteins (13). Oligosaccharide synthesis in LS-C cells is blocked in the mucin core region at the stage of addition of β3-linked galactose to the Tn antigen to form the T antigen. Native mucin purified from LS-B cells bound greater than 40-fold more galectin-3 than mucin from LS-C cells, suggesting that binding of galectin-3 depends on peripheral carbohydrate structures. Mucin from metastatic LS-Lim6 cells is highly glycosylated and bound 2-fold more galectin-3 than parental LS174T cells, which have low metastatic potential. All of the colon cancer cell lines examined also expressed galectin-3. Endogenous expression of galectin-3 paralleled the metastatic potential of these cells (10, 20).

When serum from a patient with widely metastatic colon cancer was analyzed by size exclusion chromatography, more than 70% of galectin-3 ligand was present at the void volume and chromatographed with mucin-specific carbohydrate structures, suggesting that mucin is a major circulating ligand for this protein. These results demonstrate that colon cancer mucin is a major ligand for the endogenous β-galactoside-binding lectin galectin-3. Binding of galectin-3 to colon cancer mucin may facilitate homotypic and heterotypic interactions that play a role in colon cancer metastasis.

**References**


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