Carcinoembryonic Antigen and Other Glycoconjugates Act as Ligands for Galectin-3 in Human Colon Carcinoma Cells

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ABSTRACT

Galectin-1 and galectin-3, galactoside-binding lectins with molecular weights of M, 14,500 and 31,000, respectively, are expressed in normal and malignant cells and have been implicated in regulation of cell growth, adhesion, and metastasis. We analyzed the expression of galectins in 21 cultured human colon carcinoma cell lines by immunoblotting. Galectin-1 was detected in only 7, whereas galectin-3 was found in 20 of the cell lines. KM12 cells, which express only galectin-3, were used to isolate this lectin by affinity chromatography, and the purified lectin was used to identify complementary glycoconjugates by blotting. Galectin-3 was shown to bind to human laminin, carcinoembryonic antigen, and lysosome-associated membrane glycoproteins, which are involved in cell adhesion. Galectin-3 was localized on the KM12 cell surface and colocalized with carcinoembryonic antigen. Several endogenous glycoproteins and cell surface proteins of molecular weights in the range M, 58,000 to >200,000, including carcinoembryonic antigen and lysosome-associated membrane glycoproteins, were identified as galectin-3 ligands by immunoprecipitation with and affinity chromatography on immortalized galectin-3. These data demonstrate that galectin-3 interacts with several adhesion molecules and suggest that this lectin may have a role in human colon carcinoma cell adhesion.

INTRODUCTION

Carbohydrate-recognition mechanisms have been implicated in specific cellular interactions that occur during embryonic development, during cell growth and differentiation, and during cancer metastasis (1–3). These interactions may be mediated in part by carbohydrate-binding proteins (lectins) produced by vertebrate cells and by complementary glycoconjugates that are expressed on the surface of adjacent cells or in the ECM (2–7). The most widely expressed vertebrate lectins bind galactosides (3–7). These lectins, now known collectively as “galectins” (8), constitute a family of proteins with related amino acid sequences. The two most thoroughly studied members of the galectin family have apparent molecular weights of M, 13,000–16,000 and 29,000–35,000, respectively (3–8). The lower molecular weight galectin (galectin-1) has been referred to as galabin and L-14.5 (3–5), whereas the higher molecular weight galectin (galectin-3) has been referred to as CBP35, RL-29, L-31, L-34, low affinity IgE-binding protein, and Mac-2 (5–7).

Galectins are believed to play a role in cell-cell interactions, cell adhesion to ECM, the organization of the ECM, tissue remodeling, RNA splicing/transport, growth regulation, transformation, and metastasis (3–14); however, evidence implicating galectins in these events has often been indirect. One of the most likely functions of galectins involves their interactions with complementary glycoconjugates (5–7). Indeed, several studies have demonstrated the ability of galectins to bind polylactosaminoglycan (15, 16), the ECM component laminin (17, 18), and the lamp 1 and 2, which have been demonstrated to be involved in cell adhesion (19–22). Alterations in lectin expression have been found after mitogenic stimulation of quiescent cells (23), induction of cell differentiation (24, 25), malignant transformation (12, 13), tumor progression, and acquisition of the metastatic phenotype (14).

We have previously examined galectin expression in a variety of murine and human tumor cell lines and have studied the modulation of this expression after treatment with differentiation-inducing agents (24, 25). One intriguing observation made in these studies was that while nearly all the tumor cell lines expressed the galectin-1 and several also expressed galectin-3, only one tumor cell line in these initial observations, the KM12 human colon carcinoma (HCC) cell line, expressed galectin-3 but did not express galectin-1. KM12 cells, therefore, appeared to be ideal for purifying galectin-3 and for investigating its function in the absence of galectin-1. Galectin-3 was also of interest to us because we had found that the levels of this lectin in primary tumors of patients having distant metastases (Dukes’ Stage D) were significantly higher than levels in tumors of patients without detectable metastases (Dukes’ Stages B1 and B2; Ref. 26).

The present study was designed to examine the expression of galectin-3 in HCC cells and to identify its complementary glycoconjugate ligands in these cells. After an initial analysis of the expression of galectin-1 and galectin-3 in 21 HCC cell lines, the KM12 cells were selected for further studies of galectin-3 expression on the surface KM12 HCC cell line and its interaction with several endogenous cognate glycoconjugates and purified poly-N-acetyllactosamine-containing glycoproteins. In addition, we used a recombinant murine homologue of galectin-3 to isolate and identify endogenous lectin ligands present in KM12, LoVo, and LS174T HCC cells.

MATERIALS AND METHODS

Tissue Culture. The poorly metastatic KM12-c cell line and its highly metastatic variants KM12-SM and KM12-LA were established from a primary HCC (Duke’s B2; Refs. 27 and 28). The cells were grown in a 1:1 (v/v) mixture of DMEM (GIBCO, Grand Island, NY) and Ham’s F-12 medium supplemented with 10% FBS (Hyclone Laboratories, Logan, UT), l-glutamine, nonessential amino acids, and a 2-fold concentrated vitamin solution (GIBCO) in plastic tissue culture dishes (Corning Glass, Corning, NY). The HCC-M1544 and HCC-M1410 cell lines (29) were maintained in α-MEM with 10% FBS, nonessential amino acids, vitamins, 2 mM l-glutamine, and 1.5% pyruvate. The M1544 and HCC-M1410 cell lines (29) were maintained in α-MEM with 10% FBS, nonessential amino acids, vitamins, 2 mM l-glutamine, and 1.5% pyruvate. The MOSER, GEO, HCT116, RKO, C, CBS, and FET cell lines (30, 31) were grown in McCoy’s medium containing 10% FBS, 1.5% pyruvate, 0.6% amino acids, and 2 mM l-glutamine. SW480 and SW620 were obtained from the American Type Culture Collection (Rockville, MD) and grown in L-15 medium with 10% FBS. DLD-1, LoVo, Caco-2, LS174T, SW480, HCT115, and HT-29 were also obtained from the American Type Culture Collection but...
were grown in DMEM/F-12 medium containing 10% FBS with (Caco-2 and LS174T) or without (LoVo, SW403, HCT15, and HT-29) 1% nonessential amino acids. MIP-101 (32) was maintained in RPMI 1640 with 10% FBS. The DiFi HCC cells (33) were grown in DMEM/F-12 medium containing 10% FBS.

**Metabolic Labeling of Proteins and Glycoproteins.** Cell monolayers were incubated overnight in medium containing 50 µCi/ml 35S-Trans-label (a mixture of 35S-labeled methionine and cysteine; >1000 Ci/mmol; ICN BioMedicals, Irvine, CA) to label proteins or for 24 h in glucose-free RPMI (GIBCO) containing 10% dialyzed FBS and a mixture of both [3H]lactose and [3H]glucosamine (20 µCi/ml and 25 Ci/mmol, respectively; ICN) to label glycoproteins.

**SDS-PAGE and Immunoblotting.** SDS-PAGE was performed as described previously by Lotan et al. (34). Unlabeled proteins were detected by staining with Coomassie Brilliant Blue (Sigma Chemical Co., St. Louis, MO), whereas radiolabeled proteins were detected by fluorography with Entensify (New England Nuclear, Wilmington, DE). Prestained molecular weight markers were purchased from Bio-Rad, and unstained markers were purchased from Sigma. HCC cell extracts or purified lectins were immunoblotted as described elsewhere (24, 25). The following anti-lectin antibodies were used: (a) an affinity-purified polyclonal antiserum raised in rabbits against galectin-1 purified from human placenta (34); and (b) a rat monoclonal antibody mAb M3/38 raised against the murine macrophage antigen Mac-2 (mouse galecitin-3; Refs. 35 and 36; Boehringer Mannheim, Indianapolis, IN). The following anti-CEA antibodies were used: (a) a murine mAb 7F, raised against CEA from a liver metastasis of a colon carcinoma (37), which was provided by Dr. James Chan (M. D. Anderson Cancer Center, Houston, TX); (b) a murine mAb A93 raised against a human gastric carcinoma cell line TMK-1 that recognizes both CEA and NCA (38), which was provided by Dr. Eiichi Tahara (Hiroshima University, Hiroshima, Japan). Both mAbs were used to detect CEA by immunoblotting.

**Immunoprecipitation of Cell Surface Radiolabeled Galectin-3.** KM12-c cells were radiolabeled and then lysed as described elsewhere (25). Aliquots of the 125I-cell-surface-labeled cell lysates (1 µg protein) were precleared after an incubation at 4°C with protein G-Sepharose 4B (Zymed, South San Francisco, CA), followed by centrifugation and collection of the supernatant. Samples of the supernatant were then incubated for 3 h with anti-galectin-1 or anti-galectin-3 antibodies at 4°C then with 75 µl protein G-Sepharose 4B for 30 min at 4°C with vigorous shaking. After five washes in a wash buffer (50 mM Tris-HCl (pH 8.0), 0.5 mM NaCl, 0.5% deoxycholate, 0.5% Triton X-100, 0.1% SDS, 5 mM EDTA, and 0.02% sodium azide) and one wash in lysis buffer, the Sepharose beads were boiled in an SDS-PAGE sample buffer, and the soluble material was analyzed by SDS-PAGE and subjected to autoradiography at −70°C.

**Identification of Endogenous Lectin Ligands by Coimmunoprecipitation with Galectin-3.** The procedure described by Rosenberg et al. (39) was used with some modifications. Aliquots containing 1 mg of protein from KM12-c cell lysates labeled metabolically with [3H]glucosamine and [3H]lactose or samples of lysates prepared from 125I-cell-labeled protein were precleared after an overnight incubation at 4°C with protein G-Sepharose 4B. Samples were then incubated for 3 h with an anti-galectin-3 mAb at 4°C and then with 75 µl protein G-Sepharose 4B for 30 min at 4°C with vigorous shaking. After five washes in a buffer containing 50 mM Tris-HCl (pH 8.0), 0.5 mM NaCl, 0.5% deoxycholate, 0.5% Triton X-100, 0.1% SDS, 5 mM EDTA, and 0.02% sodium azide; one wash in lysis buffer; and one elution with the above solution containing also 0.5 mM sucrose, the putative radiolabeled ligands, which remained bound to the immunoprecipitated galectin-3, were eluted by incubating the precipitate in 0.5 mM lactose in lysis buffer. After centrifugation in the microfuge, the supernatant fraction was collected, the precipitate was boiled in SDS sample buffer, and the eluted material was collected after centrifugation. Samples of the material eluted from the immunoprecipitate with sucrose, lactose, or SDS sample buffer were subjected to electrophoresis on 4 to 20% gradient gels (Bio-Rad).

**Lectin Binding to Polylactosaminoglycan-containing Glycoproteins.** Purified murine EHS laminin was purchased from Collaborative Research. Human laminin was purchased from Telios Pharmaceuticals (San Diego, CA). CEA was purified from a liver metastasis of a colon carcinoma as described by Krupye et al. (40). Human lamp-1 and lamp-2 were purified from chronic myelogenous leukemia cells as described by Carlson et al. (41). Each glyco-
were washed once and then resuspended in 1 ml DPBS prior to analysis with a Becton Dickinson flow cytometer.

The binding of galectin-3 to the surface of intact KM12-c cells was analyzed by incubating washed, resuspended cells in DPBS containing 1 mg/ml BSA prior to their fixation in 2% paraformaldehyde for 30 min on ice. The cells were then washed and resuspended in DPBS-BSA at a concentration of 10^6 cells/ml. They were then pelleted and resuspended in 100 µl DPBS-BSA containing 100 µg/ml biotinylated galectin-3 with or without 0.3 M lactose and incubated at room temperature for 30 min. Following this, the cells were washed once in DPBS-BSA with or without lactose and then incubated for 30 min at room temperature with FITC-avidin (Sigma) at 15 µg/ml in DPBS-BSA again with or without lactose. The cells were then washed and resuspended in 1 ml DPBS for analysis.

Binding of Neoglycoenzymes to the Surface of KM12 Cells. KM12-c and KM12-SM cells were grown in 24-well dishes for 24 h after trypsinization. Binding studies were carried out at 4°C essentially as described by Gabius et al. (43, 44). Briefly, monolayers were washed three times with a wash buffer (Hank’s balanced salt solution containing 20 mM HEPES). Neoglycoenzymes were prepared using β-galactosidase from Escherichia coli as described elsewhere (44) and then incubated on the monolayers for 30 min at 4°C in a final volume of 200 µl. After being rinsed quickly three times with wash buffer, the cells were incubated in 200 µl of 100 mM HEPES buffer (pH 7.0) containing 0.5% Triton X-100, 150 mM NaCl, 2 mM MgCl₂, 0.1% NaN₃, 0.1% BSA, and 1.5 mM chlorophenol red-β-D-galactopyranoside (Boehringer-Mannheim) for 1 h in the dark at 37°C. The enzymatic reaction was stopped by the addition of 200 µl of 0.2 M glycine (pH 10.5), and the colored reaction product was quantitated with a spectrophotometer at 590 nm. Specific binding of the probe was calculated by subtracting the binding of unmodified enzymes at the same concentration from the total binding observed with the glycosylated enzyme. The enzymatic activity calculated in fmol was determined with activity graphs for each enzyme preparation.

Localization of Galectin-3 and CEA by Double Indirect Immunofluorescence. Cells were grown on glass coverslips (Corning Glass) for 2 days and then washed three times with PBS containing 0.02% sodium azide. The cells were then covered with 100 µl of 3% normal donkey serum (in PBS) which contained a 1:20 dilution of ascites containing the anti-CEA mAb 7F and a 1:80 dilution of B-phycoerythrin-conjugated donkey anti-rat Fab’ (Jackson Immunoresearch, Westgrove, PA) to localize galectin-3. The cells were incubated for 1 h at 4°C, washed three times with PBS-azide, and incubated for another 1 h at 4°C with 100 µl of a solution containing a 1:25 dilution of FITC-conjugated goat anti-mouse IgG to localize CEA and a 1:80 dilution of B-phycocerythrin-conjugated donkey anti-rat Fab’ (Jackson Immunoresearch, Westgrove, PA) to localize galectin-3. The cells were then washed, fixed with 3.5% paraformaldehyde in PBS, mounted in glycerol on microscope slides, and photographed using two different filters (for green or red fluorescence) in a Nikon microscope equipped with an epifluorescence device.

To quantify the degree of colocalization of galectin-3 and CEA, we used both KM12-c and LS174T cells labeled by indirect immunofluorescence using the above anti-galectin-3 antibodies and a FITC-labeled second antibody and direct immunofluorescence labeling with rhodamine-conjugated, affinity-purified polyclonal goat anti-human CEA that does not cross react with NCA. Epifluorescence in the fluorescein (galectin-3-) and rhodamine (CEA+) channels of the same groups of cell monolayers were digitally imaged by an Optronics integrating color CCD video camera (Optronics Engineering, Goleta, CA) and captured on a Macintosh Quadra 950 (Apple Computer Co., Cupertino, CA) using Photoshop version 2.0.1 software (Adobe Systems, Inc., Mountain View, CA). The two channels were superimposed in RGB mode, and the fluorescein channel was subtracted from the rhodamine channel to yield the portion of cell surface CEA that was not associated with galectin-3 (CEA+, galectin-3-). The images of the rhodamine channel (CEA+) and the (CEA+, galectin-3-) channel were converted to grayscale and imported into IP Lab (Signal Analytics Corp., Vienna, VA), where the fluorescent area of each image was measured. The percentage of fluorescence that represented CEA colocalized with galectin-3 was then calculated. At least 300 cells were analyzed by this two-color fluorescence technique for each cell line.

RESULTS

Lactose-binding Lectin and CEA Expression in HCC Cell Lines. A collection of 21 HCC cell lines established from tumors with distinct differentiation status was selected for this study (Table 1). All but one of the HCC cell lines examined expressed galectin-3, while the expression of galectin-1 was variable and restricted to 7 cell lines (SW480, SW620, HCC-M1410, MOSER, HCT116, RKO, and SW 403), most of which were derived from poorly differentiated tumors (Table 1; Fig. 1, lower panel). A 46-kDa protein that cross-reacted with the affinity-purified polyclonal antiserum to the galectin-1 but which was not recognized by the anti-galectin-3 mAb was detected in the extracts of the HCT116, RKO, C, and CBS cell lines (Fig. 1, lower panel, arrow). The nature of this protein and its relationship to the galectin family is currently unknown. There was little difference in the expression of galectin-3 among cell lines that were derived from a primary tumor (SW480) or from a metastatic lesion (SW620) of the same patient or among KM12 variant cell lines that differed in their metastatic capability (data not shown).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Derived from</th>
<th>Duke’s Stage</th>
<th>Differentiation status</th>
<th>Lecin expressed</th>
<th>CEA expression</th>
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<tr>
<td>DLD-1</td>
<td>Primary adenocarcinoma</td>
<td>Poor to moderate</td>
<td>2.7</td>
<td>2.7</td>
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<td>SW480</td>
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<td>Poor</td>
<td>1.1</td>
<td>2.9</td>
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<td>SW620</td>
<td>Lymph node met.</td>
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<td>0.85</td>
<td>2.4</td>
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<tr>
<td>LoVo</td>
<td>Metastasis</td>
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<td>0.9</td>
<td>6.4</td>
<td></td>
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<tr>
<td>Caco-2</td>
<td>Primary adenocarcinoma</td>
<td>Well</td>
<td>1.1</td>
<td>0.5</td>
<td></td>
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<tr>
<td>LS174T</td>
<td>Primary adenocarcinoma</td>
<td>Well</td>
<td>1.3</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>HT-29</td>
<td>Primary adenocarcinoma</td>
<td>Moderate</td>
<td>1.2</td>
<td>0.06</td>
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<tr>
<td>MIP101</td>
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<td>Poor</td>
<td>1.9</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>KM12-c</td>
<td>Primary adenocarcinoma</td>
<td>B₂</td>
<td>3.1</td>
<td>0.3</td>
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<tr>
<td>HCC-M1544</td>
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<td>D</td>
<td>2.1</td>
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<td>HCC-M1410</td>
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<td>D</td>
<td>2.4</td>
<td>1.3</td>
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<td>DIFI</td>
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<td>1.1</td>
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<tr>
<td>MOSER</td>
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<td>3.2</td>
<td>0.35</td>
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<td>0.7</td>
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<td>6.5</td>
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* The numbers are relative lectin or CEA levels calculated from densitometric scanning of autoradiograms of immunoblots shown in Fig. 1.

a met., metastasis.
Fig. 1. Immunoblotting of HCC cell extracts with mAb 9A3 to CEA (top) and antibodies to galectin-1 and galectin-3 (bottom). Equal amounts of cell extract protein were loaded in each lane of a 6% (top panel) or 14% (bottom panel) polyacrylamide. After electrophoresis, the proteins were transferred to nitrocellulose membranes and immunoblotted as described in “Materials and Methods.” Numbers to the right of the upper panel represent the molecular mass in kDa of standard markers. The 180-kDa protein detected in the upper panel is CEA, and the lower molecular weight proteins are probably NCA and other cross-reacting antigens. A protein cross-reactive with affinity-purified anti-galectin-1 Ab was detected in four of the HCC cell lines (bottom, arrow).

To relate the reported differentiation status of the above cell lines with a known marker of colon carcinoma differentiation, we analyzed the expression of cell-associated CEA by Western blot analysis (Fig. 1, upper panel). The mAb 9A3 revealed the expression of CEA as well as antigenically related molecules, most likely NCA in more than one-half of the cell lines. This expression was in agreement with the reported differentiation status for most of the cell lines with well or moderately differentiated cell lines expressing higher levels of this protein than poorly differentiated ones (Table 1). There appeared to be no relationship between the expression of galectin-3 and the state of differentiation of the cells, whereas the expression of galectin-1 was inversely related to the state of differentiation.

Localization of Galectin-3 on the Cell Surface. To be able to interact with glycoconjugates at the cell surface or in the ECM, galectin-3 must be present on the cell exterior. We have demonstrated that this lectin is present on the surface of KM12 cells using three different methods: (a) galectin-3 was immunoprecipitated from extracts of KM12-SM cells that had been surface radiiodinated in monolayer culture (Fig. 2, Lane B). In contrast, antibody to galectin-1 did not immunoprecipitate either a 14.5-kDa or a 31-kDa protein from the same cell extract (Fig. 2, Lane A); (b) galectin-3 was detected on the surface of KM12 cells by flow cytofluorimetric analysis of cells labeled in suspension by indirect immunofluorescence (Fig. 3A); and (c) galectin-3 was detected on the surface of KM12 cells grown as monolayers by indirect immunofluorescent labeling with anti-galectin-3 mAb followed by phycocerytin-conjugated second antibody and observation with a fluorescent microscope (Fig. 3C). Because the cells were found to express some cell-associated CEA (Fig. 1, upper panel), we analyzed the expression of this glycoprotein on the surface of the KM12 cells using a CEA-specific mAb 7F which does not recognize NCA. Both fluorescence microscopy and flow cytofluorimetry demonstrated the presence of CEA on the surface of KM12 cells (Fig. 3, B and D). Double indirect immunofluorescence performed using a rat mAb recognizing galectin-3 and a mouse mAb specific for CEA revealed extensive colocalization of the two proteins on the surface of the KM12-c cells (compare Fig. 3, C and D). Analysis of double immunofluorescent labeling by digital microphotography revealed that 60.1 ± 16.4% and 43.7 ± 8.2% of cell surface CEA had galectin-3 associated with it on the surface of KM12-c cells and LS174T HCC cells, respectively. These results suggest that galectin-3 may have the opportunity to bind to CEA at the cell surface.

Identification of Endogenous Lectin Ligands by Affinity Chromatography using Immobilized Galectin-3. Affinity chromatography on immobilized galectin-3 was used to identify endogenous lectin ligands. Because of the low yield of endogenous galectin-3 isolated from KM12 cells by affinity chromatography on immobilized asialofetuin (48 µg from 15.05 mg protein present in the 100,000 × g supernatant of an extract prepared from 6.5 × 10⁶ cells), we used recombinant murine galectin-3, which shares a very similar carbohydrate specificity with human galectin-3 (16, 45), to prepare the affinity chromatography column. Fig. 4a shows that when extracts of KM12-c cells labeled metabolically with [³H]galactose and [³H]glucosamine were passed through the immobilized galectin-3, most of the glycoproteins did not bind to the lectin (Fig. 4a). However, washing the column with 0.5 M lactose released bound glycoproteins of about 70,
110–120, 180, 200, and >200 kDa (Fig. 4a). These glycoproteins were not significantly eluted from the column by the control disaccharide sucrose at 0.5 M or by 0.3 M NaCl (data not shown). Western blotting of samples of the lactose-eluted material demonstrated the presence of lamp-1 and lamp-2 in these fractions (Fig. 4a). Similarly, CEA and what is likely NCA or a related family member could be immunoprecipitated from the same fraction using a polyclonal anti-CEA antibody (Fig. 4a).

Because the CEA level in KM12 cells was relatively low, we wished to confirm and extend the above results by a similar analysis of extracts from two other HCC cell lines, LoVo and LS174T, which express higher CEA levels than the KM12 cells do (Fig. 1). The glycoproteins bound by galectin-3 and eluted with lactose included molecules of 40 to 200 kDa, some of which were identified as lamp-1 and CEA. LoVo cells also contained NCA that bound to the galectin-3 (Fig. 4b). The LS174T galectin-3 ligands included a prominent 70–110 kDa glycoprotein of unknown identity (Fig. 4b).

Identification of Endogenous Lectin Ligands by Coimmunoprecipitation with Galectin-3. To identify endogenous cellular proteins that might be specific recognition partner(s) of endogenous galectin-3, we analyzed material coimmunoprecipitated with galectin-3 from extracts of both [3H]sugar-labeled and cell-surface 125I-labeled KM12-c cells. The immune complexes brought down by protein G-Sepharose were washed and then eluted sequentially with sucrose, lactose, and SDS-PAGE sample buffer. Fig. 5 shows that elution with the control disaccharide sucrose prior to elution with lactose liberated only small amounts of labeled material (Fig. 5). In contrast, the majority of bound proteins were eluted specifically with lactose (Fig. 5).

The glycoproteins isolated from lactose eluates of [3H]sugar-labeled cells had molecular weights of 58, 90, 116–120, 180, and >200 kDa (Fig. 5). Proteins eluted by lactose from 125I cell-surface-labeled KM12-c cell extracts exhibited similarities as well as differences with those from the metabolically labeled extracts (Fig. 5B, compare left and right panels). The cell surface proteins bound by endogenous galectin-3 had molecular masses of 38, 58, 100, 116–120, 160, 180, and >200 kDa. Final elution of the precipitates from [3H]sugar-labeled extracts with boiling SDS-PAGE sample buffer released small amounts of glycoproteins that were similar to those
eluted with lactose (Fig. 5). However, the denaturing sample buffer released distinct proteins that remained bound after lactose elution from cell surface radioiodinated cell extracts. Two prominent protein bands with molecular weights of ~94 and 140 kDa were found exclusively in this SDS eluate (Fig. 5).

**Galectin-3 Binds Laminin, CEA, Lamp-1, and Lamp-2 in a Carbohydrate-dependent Manner.** To further examine and confirm more directly the interaction between galectin-3 and possible cognate ligands, galectin-3 purified from KM12-c cells or murine recombinant galectin-3 was used for binding to glycoconjugates containing polylactosaminoglycans including purified murine laminin, human laminin, CEA, lamp-1, and lamp-2 electroblotted onto nitrocellulose membranes (Fig. 6). Galectin-3 binding to these glycoproteins was detected by anti-galectin-3 mAb (Fig. 6a). That this binding was mediated by the carbohydrate-binding site of galectin-3 was indicated by the ability of 0.1 M lactose to block lectin binding (Fig. 6b). The binding of galectin-3 to human laminin differed from its binding to mouse laminin in this assay. Although the lectin appeared to bind very well to the A chain of both human and mouse laminin, it did not bind as well to the B chains of human laminin as it did to the B chains of mouse laminin. Consequently, there was less galectin-3 bound by human laminin than there was to an equivalent amount of murine laminin. Galectin-3 also bound well to human CEA and to both lamp-1 and lamp-2.

To further investigate the interaction between galectin-3 and CEA, slot-blot analyses were performed using increasing amounts of purified human CEA immobilized on a nitrocellulose membrane and a biotinylated preparation of affinity-purified recombinant galectin-3 as a probe (Fig. 6, c and d). This revealed the dose-responsiveness of binding of the lectin to increasing amounts of CEA. In addition, galectin-3 binding to human CEA could be completely inhibited by lactose, demonstrating the carbohydrate dependence of this interaction (Fig. 6).

**Galactoside-binding Activity and Binding Sites for Exogenous Galectin-3 Are Present on the Surface of KM12 Cells.** The ability of galectins to mediate carbohydrate-recognition events involved in cell-cell and cell-ECM adhesion implies that galectin-like carbohydrate-binding activity should be detectable on the surface of intact cells. We have identified such an activity using a sensitive assay for neoglycoprotein binding to intact cells. Table 2 shows that several galactoside-containing neoglycoproteins bound to the surface of the KM12-c cells. The binding of thiogalactoside was about 5-fold higher than the binding of either lactose- or melibiose-derivatized neoglycoprotein, a finding that is consistent with the reported higher affinity of galectins for thiogalactoside than for galactosides (16). The binding of N-acetylgalactosamine-derivatized neoglycoprotein to

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Fig. 6. Carbohydrate-dependent recognition of purified polylactosaminoglycan-containing glycoproteins by galectin-3. a and b, the purified glycoproteins were run on 5% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with purified galectin-3 from KM12-c cells in the absence (a) or presence (b) of 0.1 M lactose. The blots were then incubated with anti-galectin-3 mAb followed by 125I-labeled second Ab, washed, and exposed for autoradiography. Numbers to the left of the gels represent the molecular mass of standard markers in kDa. LM (h), human laminin; LM (m), murine laminin. c to assess binding of galectin-3 to CEA, aliquots containing 1, 2, 4, or 8 μg of CEA were slot-blotted onto nitrocellulose in duplicate. The blots were incubated with biotinylated recombinant galectin-3 in the absence (A) or presence (B) of 0.3 M lactose. Lectin binding was revealed by incubation with 125I-streptavidin, washing, and autoradiography. d, the results of densitometric scanning of the autoradiograph demonstrate the quantitative difference in binding in the absence (C) and presence (D) of lactose. Binding to 1 μg of CEA in the absence of lactose was assigned an arbitrary value of 1.
the KM12-c cells was even lower than that of galactosides (Table 2). These results suggested that a galactoside-specific, lectin-like activity is present on the surface of these cells.

To determine whether galectin-3 could bind to the KM12 cell surface, cells were incubated with biotinylated galectin-3 followed by FITC-avidin. Flow cytofluorimetry (Fig. 7) revealed that galectin-3 readily binds to the KM12c cell surface. Because a significant part of this binding (about 50%) was inhibited in the presence of lactose, we concluded that the galectin-3 binding we observed was at least partially carbohydrate dependent.

DISCUSSION

A high level of binding of the Mac-2 antibody, which recognizes galectin-3 (35, 36), has been detected in small intestinal epithelial cells located at the tips of villi, whereas only a low level of reactivity was found in cells at the base of the villi (46). Furthermore, nonmalignant colonic mucosa cells adjacent to colon carcinomas were found to contain the galectin-3 mRNA and protein (47, 48). We (25, 26, 49) and others (39, 46–48, 50, 51) reported on the presence of galectin-3 in surgical specimens and HCC cell lines.

Our study shows clearly that: (a) galectin-3 is expressed in 20 of 21 HCC cell lines, irrespective of their differentiation state; (b) galectin-1 is expressed by only a few HCC cell lines, primarily poorly differentiated ones; (c) galectin-3 can bind purified lamp-1 and lamp-2 as well as endogenous lamps from HCC; (d) galectin-3 can bind CEA and most probably NCA as well; (e) galectin-3 is expressed on the surface of the KM12-c cells and appears to be colocalized with CEA; and (f) endogenous cognate ligands, including lamps, CEA, and NCA, which are distinct from those reported by others (39, 50) are present in HCC cells.

The ability of galectin-3, which is present on the surface of HCC cells to bind laminin was reported previously (51) and confirmed in the present study. This activity may play a role in cell binding to ECM, an important event in cancer cell invasion and metastasis (52). However, the mere expression of galectin-3 on the surface of a cell may not be sufficient to mediate carbohydrate-specific adhesion to laminin (53). The binding of recombinant human galectin-3 to mouse EHS laminin has also been reported (53, 54). We found that galectin-3 purified from KM12 cells binds to both human and murine laminin, although its binding was greater to murine laminin than to human laminin. The binding of galectin-3 to the B chain of murine laminin was greater than to the A chain, as found by Massa et al. (54). The difference in galectin-3 binding to the purified laminins from human and mouse suggests that it may be important to use human laminin rather than murine laminin when examining the interaction of human tumor cells with laminin. Recently, a number of cellular interactions with laminin have been shown to be dependent on the state of glycosylation of this glycoprotein (55), and a recent report has demonstrated that galectin-1 may be involved in the adhesion of cells to laminin (56).

The KM12 cells express on their surface both CEA (this study) and lamp-1 (57). Because these two glycoproteins can be recognized by galectin-3, they could serve as galectin ligands in cell-cell adhesion.

Table 2 Binding of neoglycoproteins to KM12-c HCC cells

<table>
<thead>
<tr>
<th>Saccharide on neoglycoprotein</th>
<th>Saccharide bound (fmol/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiodigalactoside</td>
<td>7.78</td>
</tr>
<tr>
<td>d-Galactopyranosylβ1-4-d-glucopyranoside (lactose)</td>
<td>1.51</td>
</tr>
<tr>
<td>d-Galactopyranosylα1-6-d-glucopyranoside (melibiose)</td>
<td>1.57</td>
</tr>
<tr>
<td>N-Acetyl-d-glucosamine</td>
<td>1.19</td>
</tr>
</tbody>
</table>

*a The indicated saccharides were bound covalently to E. coli β-galactosidase, and the neoglycoenzyme derivatives were used for binding to cell monolayers as described in "Materials and Methods."
sialoglycoproteins in a variety of different cell types and the major carriers of poly-N-acetyllactosamine (1, 19, 41). lamp-1 and lamp-2 are present on the surface of some cells, including the KM12 cell line used in this study (19, 57). Cell surface lamps have been implicated in cell adhesion and metastasis of KM12 cells by other studies (57, 62). The ability of galectin-3 to bind lamp-1 and lamp-2 suggests that these molecules may also be functional ligands for this lectin. Previously, it was reported that galectin-1 was able to bind lamps from Chinese hamster ovary and ovarian carcinoma cells (20, 22). In ovarian carcinoma cells, surface lamps were shown to mediate adhesion to polymerized galectin-1 (21, 22). Here, we report that human galectin-3 can also interact with lamps. A potential role for KM12 cell surface lamps in adhesive interactions with E-selectin has been demonstrated recently (62). We now propose that galectin-3 may also be involved in these adhesive interactions.

The ligands for galectin-3 that were detected among total cellular glycoconjugates labeled metabolically with [3H]glucosamine and [3H]galactose by coimmunoprecipitation with endogenous galectin-3 included glycoproteins of apparent molecular masses of 58 and 90 kDa (Fig. 4, a and b), which may be similar to those identified previously in other HCC cell lines based on metabolic labeling with [35S]methionine (39) or immunoprecipitation/immunoblotting (48). Rosenberg et al. (39) identified two related endogenous glycoproteins, M2BP-1 (98 kDa) and M2BP-2 (70 kDa) as ligands for galectin-3 in five of seven colon carcinoma cell lines they examined. More recently, a Mac-2 binding protein (Mac-2-BP) has been cloned and sequenced by Koths et al. (50) and found to exhibit a significant homology to the extracellular domain of the type I macrophage scavenger receptor as well as to the lung tumor antigen, L3. Surprisingly, the 58- and 90-kDa molecules isolated by coimmunoprecipitation with endogenous galectin-3 were not bound by the immobilized recombinant galectin-3. It is possible that coimmunoprecipitation is more sensitive than affinity chromatography, in that the former may detect ligands with lower affinity as a result of the formation of multivalent anti-galectin/galectin-ligand complexes.

In addition to the above ligands, we detected by both coimmunoprecipitation and affinity chromatography additional ligands for galectin-3 that were not reported in HCC before. These included glycoproteins of molecular masses ranging between 120 and >200 kDa. The identity of several >200-kDa ligands found in the KM12 cells is still unknown. However, a further analysis of affinity-purified material by Western blotting and immunoprecipitation with specific antibodies identified lamp-1, lamp-2, CEA, and what may be NCA as galectin-3 ligands in three HCC cell lines. Rosenberg et al. (39) did not detect any of these fairly abundant glycoproteins as galectin-3 ligands in the HCC cells that they analyzed. The reason may be that they labeled cells with [35S]methionine, whereas we labeled cells with [3H]galactose and [3H]glucosamine. In addition, we used direct affinity chromatography with immobilized galectin-3, whereas Rosenberg et al. (39) used coimmunoprecipitation with galectin-3 for their isolation procedure.

To be able to mediate cell-cell and cell-ECM interactions, galectin-3 should be present on the cell surface, it should be able to bind exogenous glycoconjugates, and the cells should express complementary glycocoyjugates that could serve as binding sites for galectin. The presence of different carbohydrate-binding proteins on the surface of HCC cells has been shown previously (63). That galectin-3 is present on the cell surface and was demonstrated by several methods including cell surface radiiodination, immunofluorescence microscopy, and flow cytometry; that galactoside-specific, lectin-like activity is present on the surface of intact KM12 cells was indicated by the binding of thiodigalactoside-containing neoglycoproteins; that exogeous biotinylated galectin-3 can bind in a lactoside-inhibitable fash-
LECTINS AND GLYCOPROTEINS IN COLON CARCINOMA


Carcinoembryonic Antigen and Other Glycoconjugates Act as Ligands for Galectin-3 in Human Colon Carcinoma Cells

David W. Ohannesian, Dafna Lotan, Peter Thomas, et al.


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