Disialosyl Galactosylgloboside as an Adhesion Molecule Expressed on Renal Cell Carcinoma and Its Relationship to Metastatic Potential

Makoto Satoh, Kazuko Handa, Seiichi Saito, Satoru Tokuyama, Akihiro Ito, Noriomi Miyao, Seiichi Orikasa, and Sen-itiroh Hakomori

Department of Urology, Tohoku University School of Medicine, Sendai, Japan [M. S., S. S., S. T., A. I., S. O.]; The Biomembrane Institute and Department of Pathobiology, University of Washington, Seattle, Washington 98119-4237 [K. H., S. H.]; and Department of Urology, Sapporo Medical College, Japan [N. M.]

ABSTRACT

Aberrant glycosylation expressed in specific types of human cancer may define stage, direction, and fate of tumor progression. Well-studied examples are expression of sialosyl-Lewis$^a$ or sialosyl-Lewis$^b$ in colorectal carcinoma and histo-blood group A and H/Leg in lung cancer. In renal cell carcinoma (RCC), expression of sialosyl-Lewis$^b$ has no correlation with metastatic potential. Clinico-pathological studies have revealed that the degree of expression of disialosyl galactosylgloboside (DSGG) and monosialosyl galactosylgloboside is correlated with metastatic potential (to lung and lymph nodes) of RCC and inversely correlated with patient survival.

In the present study, we compared the adhesion of RCC lines to sections of various tissues measured by Stamper-Woodruff assay and other similar assays under dynamic flow conditions. Of the eight RCC lines tested, only TOS-1 (which expresses DSGG) bound strongly to lung tissue sections. TOS-1 did not bind to sections of liver, kidney, or lymph nodes. In the same eight RCC lines, we also compared expression of DSGG and monosialosyl galactosylgloboside (reflected by reactivity with RM1 and RM2), overall ganglioside patterns, and correlation with lung tissue-binding ability. Under both static and dynamic flow conditions, the binding of TOS-1 cells to lung alveolar tissue was correlated with their DSGG expression, i.e., the binding was inhibited by RM2 but not by RM1. This binding was also inhibited by sialidase but not by EDTA (i.e., it was CA$^+$/dependent).

The other seven cell lines (TOS-2, TOS-M, SMKT-R1, -R2, -R3, and -R4, and ACHN), which do not express DSGG, showed much weaker adhesion to lung tissue. None of the eight cell lines showed E- or P-selectin-dependent adhesion. These results suggest the existence of a yet-uncharacterized sialoadhesive receptor that specifically recognizes DSGG. This receptor could be the binding target in RCC metastasis to lung.

INTRODUCTION

Aberrant glycosylation is a common denominator of essentially all animal and human cancers (1). The correlation between aberrant glycosylation and metastasis has been studied extensively in animal models (2–7). In certain types of human cancer, the degree of expression of a specific GSL$^b$ or carbohydrate epitope in the original tumor is clearly correlated with metastatic potential and clinical survival rate. For example, in various lung carcinomas (excluding small cell carcinoma), expression of histo-blood group A antigen (8) and its precursor H/Leg$^b$/Le$^a$ showed negative and positive correlation, respectively, with patient survival rate (9). Expression of SLe$^b$ or SLe$^a$ in primary colonic cancer is strongly correlated with patient survival rate (10, 11). A similar trend was seen for sialosyl-Tn expression in colonic cancer, i.e., if the antigen was not expressed in primary tumor, 5-year survival was 100%; if the antigen was expressed, survival was <70% (12). On the other hand, expression of aberrant glycosylation varies tremendously, depending on tumor type and stage (13).

RCC is a highly metastatic type of cancer showing preferential blood-borne metastasis to lung as compared to liver or kidney or distant lymph nodes. SLe$^a$ expression in RCC was not correlated with metastatic potential or degree of malignancy in our previous clinicopathological study (14). The only carbohydrate markers showing clear correlation with RCC metastasis and malignancy were MSGG and DSGG (Table 1), defined respectively by mAbs RM1 and RM2 (15).

We now report: (a) reactivities of eight different RCC lines with various mAbs directed to SLe$^a$, SLe$^b$, MSGG, and DSGG; (b) adhesion of the cell lines to frozen sections of lung, liver, kidney, and lymph node tissue, according to the Stamper-Woodruff assay; (c) adhesion of the cell lines to lung tissue sections under dynamic flow conditions; and (d) correlation of adhesion as in b and c with expression of defined carbohydrate epitopes. Our findings indicate that strong adhesion of cell line TOS-1 to lung tissue, a possible basis for lung cancer metastasis, is ascribable to the high expression of DSGG in these cells.

MATERIALS AND METHODS

Cell Lines and Cell Culture. Cell line TOS-1 was derived from a back metastatic site (spindle cell type and clear cell mixture type RCC) of a 62-year-old man. TOS-2 was derived from an axillary s.c. metastatic site (clear cell RCC) of a 78-year-old man. Both these cell lines grew well in culture, for over 20 months. SMKT-R1, -R2, -R3, and -R4 were obtained from a primary lesion of human RCC (16, 17). ACHN (purchased from Dainihonseiyaku Co., Tokyo, Japan) was from a malignant pleural effusion of a man with metastatic RCC. Each of these cell lines was cultured in MEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS and containing 0.1% penicillin (1000 units/ml) and 10 mg/ml streptomycin (Life Technologies, Inc.) in a humidified atmosphere of 5% CO$_2$ at 37°C.

Normal HUVEC were obtained from Clonetics Corp. (San Diego, CA) and cultured in medium containing endothelial cell growth factor.

Promyelocytic leukemia HL60 and histiocytic leukemia U937 cell lines were obtained from American Type Culture Collection (Rockville, MD) and cultured in RPMI supplemented with 10% FCS.

GSL Extraction. Cell lines were cultured until total cell mass was >500 mg (~10$^8$ cells). Cells were washed three times in PBS and harvested by centrifugation. GSLs were extracted as described previously (13). Briefly, a crude GSL fraction was extracted from cell pellets by homogenization and filtration with isopropanol:hexane:water (55:25:20) and CM (2:1, 1:1, and 1:2 v/v). The sample was divided into upper and lower phases by Folch’s partition (18). The upper phase was dialyzed against distilled water for 2 days and then divided into upper neutral and acidic fractions by DEAE Sephadex A25 column chromatography (19). Acidic fractions containing gangliosides extracted from cells and standard gangliosides were placed on high-performance TLC plates (Whatman HPKF, Clifton, NJ) using a microsyringe. The solvent for high-performance TLC was CMW 50:40:10 containing 0.05% CaCl$_2$. The detection reagent was orcinol-sulfuric acid.

TLC Immunostaining. This was performed on TLC plates (Whatman) by a modified version of Magnani’s procedure (20). Total ganglioside fractions were applied on TLC plates for chromatography using a solvent system of CMW 50:40:10 containing 0.05% CaCl$_2$. Plates were air-dried and immersed

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Table 1 Structures and defining mAbs of MSGG and DSGG

<table>
<thead>
<tr>
<th>Structure</th>
<th>Defining mAb</th>
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<tbody>
<tr>
<td>MSGG</td>
<td></td>
</tr>
<tr>
<td>Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1Cer</td>
<td>RM1</td>
</tr>
<tr>
<td>3</td>
<td></td>
</tr>
<tr>
<td>NeuAcα2</td>
<td></td>
</tr>
<tr>
<td>DSGG</td>
<td></td>
</tr>
<tr>
<td>Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1Cer</td>
<td>RM2</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
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<tr>
<td>NeuAcα2</td>
<td>NeuAcα2</td>
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</table>

in 0.5%-poly(isobutyl-metacrylate) (high molecular weight; Aldrich Chemical Co., Milwaukee, WI) in ether for 1 mm, blocked with 5% BSA in PBS for 1 h at room temp, and then reacted overnight with mAb RM1 or RM2 at 4°C. Plates were washed, incubated with biotinylated secondary antibody for 1 h, incubated with Vector avidin-biotin solution for 30 min, and stained with 3',3-diaminobenzidine.

Flow Cytometry. Cells were incubated with mAb RM2, RM1, SNH4 (IgG3) (21), or CA19-9 (10 [g/ml; Ref. 22) in PBSSA (PBS containing 1% BSA and 0.1% sodium azide) for 90 mm on ice. Cells were washed and fixed with 1% paraformaldehyde in PBS. Non-immune mouse IgG or IgM was used as negative control. Cells (10^6) were analyzed by cytometry (EPICS Profile; Coulter Electronics Corp., Hialeah, FL), and the percentage of positivity was expressed relative to negative control.

Cell Adhesion in a Static System. Surgically resected normal human lung, kidney, liver, and lymph node tissues were donated by the Department of Thoracic Surgery, Institute of Development, Aging, and Cancer, School of Medicine, Tohoku University. Frozen section assay was adapted from the procedure of Stamper and Woodruff (23, 24). Tissues were rapidly frozen to −80°C in OCT compound. Cryostat sections (8 μm) were mounted on silancocoated microslides near one end, air-dried for ~60 min, and stored at −20°C until use in a tightly closed slidebox. Prior to the cell adhesion assay, 5% BSA containing PBS was layered on the sections for 1 h. Cell lines TOS-1, ACHN, and SMKT-R-4 were used for the assay. For inhibition of cell adhesion, mAbs RM1 and RM2 were preincubated with cultured tumor cells at 25–50 μg/ml for 30 min at room temp prior to the adhesion assay. For controls, TOS-1 cells were treated with 10 μg/ml mouse IgM (Zymed Laboratories Inc., San Francisco, CA), or with Arthrobacter ureafaciens sialidase (Sigma Chemical Co.; 0.1 unit/ml), in PBS at 4°C for 30 min. After treatment, cell viability was tested using trypan blue.

The assay was initiated by layering 100 μl of cell suspensions (1 × 10^6/ml) at 4°C in assay medium (MEM containing 1% FCS ± 5 mm EDTA) onto the sections. Slides were rotated on a platform at 70 rpm for 30 min at room temp. Unbound cells were decanted and washed by dipping the slides repeatedly in cold PBS. Sections were then fixed in methanol, stained with hematoxylin/eosin, and examined under a light microscope. Cells bound to lung alveolar tissue were counted visually (×200; from three to five separate sections each from various regions of the lung, five fields/section; assay was performed in duplicate). Data points represent mean ± SE for regions of the lung.

Cell Adhesion in a Dynamic Flow System. A parallel-plate laminar flow chamber connected to an infusion pump was constructed and used as described previously (25, 26). Briefly, the chamber consisted of a glass plate and a parallel, transparent plastic surface. A silane-coated glass slide mounted with lung tissue, or a slide on which HUVEC were grown to a confluent monolayer, was placed in the chamber. Laminar flow with defined wall shear stress was achieved by manipulation of the pump. Suspended cells with or without prior treatment with mAb or EDTA were passed through the chamber. Cell movements were observed under inverted phase contrast microscope and recorded by a time-lapse videocassette recorder. Adhesion was observed as rolling followed by stopping of cells. Numbers of cells bound during a 3-min interval at various shear stresses (1.7–7.7 dynes/cm^2) were counted from several fields recorded on videotape. Shear stress (T) was calculated by the equation of Lawrence et al. (25).

Adhesion of Glycolipid-coated Fluorescent Beads to Lung Tissue. To confirm the direct interaction of DSGG with lung tissue, fluorescent polystyrene beads (FluoSpheres, 4.0 μm diameter; Molecular Probes, Eugene, OR) were washed in ethanol. Various glycolipids (20 μg) were dissolved in CM 2:1 and mixed with ~5 × 10^6 beads resuspended in 40 μl ethanol. Mixtures were dried under nitrogen stream, and the residue was suspended in 100 ml of 0.1 M NaCl containing 0.3% gelatin. Tissue sections on glass plates were preincubated with 0.3% gelatin in 0.1 M NaCl at room temp for 1 h. Sections were then placed in the glycolipid-fluorescent bead suspension and incubated at room temp for 20 min in a moist chamber on a gyratory shaker (50 rpm). Sections were washed by dipping in 150 ml PBS 18 times and stained in H&E. Beads adhering to tissue were observed by fluorescence microscopy.

Selectin-dependent Adhesion Assay. Soluble fusion proteins of human E and P-selectin were prepared as reported previously (27). Briefly, Chinese hamster ovary DG44 cells (donated by Dr. L. A. Chasin, Columbia University, NY) were cotransfected with pCDM8 containing E- or P-selectin and human IgG1 Fc portion (donated by Dr. Brian Seed, Massachusetts General Hospital, Boston, MA; Ref. 28) and pSV2/hfr (American Type Culture Collection, Rockville, MD), and amplified with methotrexate (Sigma). Ninety-six-well flat-bottomed microtiter plates (Falcon, Becton-Dickinson, Lincoln Park, NJ) were coated with goat antihuman IgG antibody (50 μl of 5 μg/ml solution/well). Wells were washed three times with PBS, blocked with 5% BSA in PBS, coated with 100 μl of selectin fusion protein (culture supernatant containing ~1 μg/ml fusion protein) or human IgG1 in PBS for 2 h at room temperature, and washed three times with binding buffer (HBSS, 1% FCS, 10 mM HEPES (pH 7.4), and 0.05% NaN3). [3H]Thymidine-labeled tumor cells were detached with EDTA, washed, resuspended in binding buffer, added to each well, and allowed to bind for 20 min at room temperature. After washing out unbound cells, bound cells were detached with trypsin EDTA and counted by scintillation counter.

We also tested E-selectin-dependent binding activity using HUVEC. Cells were grown to confluency in 96-well plates and stimulated with TNF-α (Genzyme Corp., Cambridge, MA) at 100 units/ml. After 4 h incubation at 37°C, cells were washed with binding buffer. [3H]Thymidine-labeled tumor cells were added to the HUVEC and assayed for binding activity as described above. HUVEC without TNF-α stimulation were used as a negative control.
RESULTS

GSL Expression Patterns of Eight RCC Lines. Orcinol-sulfuric acid staining patterns of GSLs from seven of the cell lines are shown in Fig. 1, left panel. Greatly increased concentration of the band corresponding to MSGG and slightly increased concentration of DSGG were observed in cell lines derived from metastatic deposits.

Fig. 2. Reactivities of various RCC lines with mAbs directed to SLe\(^a\), SLe\(^b\), and globo-series gangliosides. Reactivities were determined by flow cytometry and expressed as the percentage of positivity relative to control murine IgG. RM2 reactivity was determined by FACScan (Becton-Dickinson); other reactivities were determined by EPICS analyzer. The expression of SLe\(^b\) was determined by mAb SNH4 (IgG\(_3\)); SLe\(^a\) was determined by CA19-9 (IgG1); MSGG was determined by RM1; and DSGG was determined by RM2. Control reactivities of HL60 cells (for SNH4) and Colo205 (for CA19-9) were both 100%.

TLC immunostaining with RM1 revealed increased MSGG in cell lines ACHN and TOS-2 (Fig. 1, middle panel, Lanes 4 and 5). Immunostaining with RM2 revealed increased DSGG in TOS-1 and TOS-M (Fig. 1, right panel). No clear expression of globo-series gangliosides reactive with RM1 or RM2 was observed for SMKT-R-1, -3, or -4 (Fig. 1, middle and right panels, Lanes 6–8).

Fig. 3. Binding patterns of RCC lines to human normal lung tissue sections. a. comparative adhesion of TOS-1, ACHN, and SMKT-R-4. Frozen sections prepared by cryostat were placed on an objective glass plate and incubated with suspensions of RCC lines (1 \times 10^5 cells) in 100 \mu l of MEM containing 1% FCS for 30 min. Cells were harvested with 0.02% EDTA, and 10^6 cells/ml suspensions in MEM were prepared. Bar, 100 \mu m \times 100. Note that only TOS-1 adhered strongly. b, effects of mAbs, EDTA, and sialidase on binding of TOS-1 to lung sections. Cells were incubated with RM1 or RM2 (20 \mu g/ml) for 30 min, treated with sialidase (0.1 unit/ml) for 30 min, or treated with EDTA (5 mM), and then placed on tissue sections. Only RM2 and sialidase inhibited binding. EDTA had no effect on adhesion, even at increased concentration (10 mM).
Reactivities of RCC Lines with mAbs Directed to Four Sialosyl Epitopes. Five RCC lines were analyzed by flow cytometry for their reactivity with four mAbs directed to SLe\(^a\), SLe\(^b\), MSGG, and DSGG. In each case, reactivity was expressed as the percentage of positive cells relative to negative control (mouse IgG), as shown in Fig. 2. None of the RCC lines reacted with anti-SLe\(^a\) CA19-9 or anti-SLe\(^b\) SNH4, whereas Colo205 showed 100% reactivity with CA19-9 and HL60 showed >99% reactivity with SNH4 (data not shown). ACHN and TOS-2 cells reacted with anti-MSGG RM1. Only TOS-1 reacted strongly with anti-DSGG RM2. Intensity of reactivity of TOS-1 cells with RM2 was much stronger than that of TOS-2 cells with RM1. TOS-1 showed no reactivity whatsoever with RM1, and TOS-2 did not react with RM2. SMKT-R-3 and -4 did not react with any of the mAbs.

Adhesion of RCC Lines to Frozen Tissue Sections by Stamper-Woodruff Assay. RCC shows a higher incidence of blood-borne metastasis to lung compared to other organs, in addition to the regional lymph nodes metastasis, which is common to all carcinomas. To test the possibility of preferential adhesion of RCC to lung sections as a basis for preferential lung metastasis, we studied adhesion of various RCC lines to frozen sections of lung (Fig. 3) and kidney and liver (Fig. 4) by Stamper-Woodruff assay. Only TOS-1 (but not TOS-2, ACHN, or SMKT-R-4; Fig. 3a) adhered strongly to the lung sections, particularly at alveolar (including perialveolar microvascular) tissues. A typical TOS-1 adhesion pattern is shown in Fig. 3a, upper panel. TOS-1 cells preincubated with mAb RM2 or treated with sialidase showed no adhesion to lung (Fig. 3b, RM2). Adhesion of TOS-1 to lung was not reduced by mAb RM1 or EDTA (Fig. 3b).

Adhesion of DSGG-coated Fluorescent Beads to Lung Sections. DSGG-coated beads showed higher binding to lung tissue than did MSGG- or paragloboside-coated beads under the conditions described in "Materials and Methods." The number of beads bound was too small for quantitative determination.

Selectin-dependent Adhesion of RCC Lines, HL60, and U937. E- and P-selectin-dependent adhesion of three RCC lines (TOS-1, SMKT-R-4, and ACHN) was compared to that of HL60 and U937 (Fig. 7). None of the RCC lines showed appreciable adhesion to E-selectin (Fig. 7A), P-selectin (Fig. 7B), or TNF-α-stimulated HUVEC (Fig. 7C). Positive control cells (HL60 and U937) adhered strongly to E- and P-selectin and activated HUVEC (Fig. 7).


discussion

Occurrence of tumor cell metastasis depends on many mechanisms and factors, including: (a) decreased adhesion between cells within the tumor cell mass through intercellular adhesion molecules such as E-cadherin (29, 30) and Le\(^a\) (31); (b) interaction (adhesion) of tumor cells through their adhesion receptors (integrins and Ig family receptors) with extracellular matrix (e.g., basement membrane) or surfaces.
of other cell types (32); (c) ability of tumor cells to activate platelets and endothelial cells to elicit expression of selectins and other adhesion molecules (33); (d) tumor cell interaction through galectin that recognizes target cell surface carbohydrate (34, 35); (e) increased motility of tumor cells for infiltration of normal tissues (36); and (f) their enhanced proteolytic or glycosidase activity (37, 38). Processes e and f may depend on specific glycosylation pattern, e.g., enhanced GlcNAc-V (41) or Lea expression (8, 42), or aberrant glycosylation in polylactosamine of lysosomal membrane protein-1 (43). Glycosylation-dependent changes in metastatic potential have been well documented in a variety of experimental tumors (37, 38, 43). A possible correlation of metastatic potential with SLea/SLea expression, the target of E- and P-selectin in tumor cells, is a major focus of recent studies (10, 39, 40). Expression of SLea and SLea in primary tumors is closely correlated with invasiveness (40), metastasis (44), and patient survival (10, 11, 45, 46). This trend is not observed in RCC (14).

Glycosylation patterns of tumor cells and their correlation with malignancy vary extensively, depending on the stage and type of tumor. In our previous studies on seminoma, we found that the degree of expression of galactosylgloboside (Galβ1→3-GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1Cer; identical to “stage-specific embryonic antigen-3”) was inversely correlated with metastatic potential, i.e., expression of this antigen inhibits metastasis (47). Similarly, elevated expression of SLea in RCC indicates a higher grade of differentiation and is inversely correlated with metastatic potential and malignancy (14). This is in striking contrast to colonic or gastric cancers in which SLea expression is directly correlated with metastatic potential (10, 39, 40). Our recent clinicopathological studies on RCC revealed that expression of sialosyl galactosylgloboside (globo-series ganglioside; stage-specific embryonic antigen-4), defined by mAbs RM2 and RM1, is closely related to the degree of metastatic potential.3 mAb RM2 defines the novel ganglioside DSGG, whereas mAb RM1 defined previously known MSGG (Ref. 15; Table 1).

We established several RCC lines and measured their reactivity with various anti-ganglioside mAbs. Binding of these RCC lines with frozen sections of lung, kidney, liver, and lymph node was also examined. RCC line TOS-1 was highly reactive with anti-DSGG mAb RM2. Adhesion of ACHN and SMKT-R-4 to lung was much weaker than that of TOS-1. This weak adhesion was not abolished by treatment with sialidase or anti-MSGG mAb RM1. Only TOS-1 adhesion was inhibited by treatment with sialidase or mAb RM2. This inhibition was also seen under dynamic flow conditions.

Adhesion of TOs-1 cells to lung tissue is clearly mediated by DSGG, not RM1; (d) the adhesion was also inhibited by sialidase treatment of TOs-1 cells; and (e) fluorescent beads coated with DSGG showed preferential adhesion to lung tissue. Inhibition by liposomes containing DSGG is theoretically possible, but in previous studies along this line, nonspecific adhesion of liposomes to tissue sections obscured the results. Adhesion of TOs-1 to lung sections is not affected by EDTA, which rules out the possibility of involvement of C-type (calcium-dependent) lectins (48) or known sialoadhesins (49, 50). The presence of αl→4Gal linkage at the internal carbohydrate chain produces a unique conformation and orientation of the nonreducing terminus of disialylated Galβ1→3GalNAc structure (15). Therefore, the receptor molecule present on alveolar lung tissue that recognizes this disialosyl structure is presumably highly specific and essential for initiating the process of RCC metastasis to lung. The recognition could be based on a novel, specific "sialoadhesin" different from known types (49, 50). Alternatively, it could be based on carbohydrate-carbohydrate interaction, as has been observed between Gg3Cer and GM3 gangliosides (3, 51), between KDN G@3-Gg3Cer interaction is not EDTA sensitive and does not require bivalent cation.9 Identification of such a molecule expressed on normal lung tissue is essential for understanding the mechanism of RCC metastasis, and studies along this line are underway.

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