Ganglioside DSGb5, preferred ligand for Siglec-7, inhibits NK cell cytotoxicity against renal cell carcinoma cells

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In renal cell carcinoma (RCC), the presence of higher gangliosides correlates with systematic metastasis. Disialosyl globopentaosylceramide (DSGb5) was identified previously as one of the major gangliosides from RCC tissues. Siglec-7 (sialic acid-binding Ig-like lectin-7), expressed on natural killer (NK) cells as an inhibitory receptor, has a striking preference for internally branched α2,6-linked disialic gangliosides such as DSGb5. To clarify the functional role of DSGb5 in RCC metastases, we have investigated whether DSGb5 expressed on RCC cells can modulate NK cell cytotoxicity in a Siglec-7-dependent manner. The binding activity of RCC cells to Siglec-7-Fc fusion protein was specifically inhibited by anti-DSGb5 monoclonal antibody and transfection of siRNA for ST6GalNAcVI (synthetase of DSGb5). These observations showed that Siglec-7-Fc fusion protein specifically bound to DSGb5 expressed on RCC cells. In contrast, the sialic acid-binding site of Siglec-7 on NK cells was masked by cis interactions with endogenous sialoconjugates at the cell surface, but it could be unmasked by sialidase treatment of the NK cells. Following sialidase treatment of NK cells, NK cell cytotoxicity against RCC cells was significantly decreased relative to cells with low DSGb5 expression. These findings indicate that such NK cell cytotoxicity against RCC cells could be inhibited by the interaction between Siglec-7 on effector cells and DSGb5 on target cells. The results of the present study suggest that DSGb5 expressed on RCC cells can downregulate NK cell cytotoxicity in a DSGb5-Siglec-7-dependent manner and that RCC cells with DSGb5 create favorable circumstance for their own survival and metastases.

Keywords: DSGb5/ganglioside/NK cell cytotoxicity/renal cell carcinoma/Siglec-7

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NK cells and that Siglec-7 is constitutively masked on NK cells, but after unmasking with sialidase it can interact with GD3 on target cells and inhibit NK cell cytotoxic activities (Nicol et al. 2003). We have studied the binding specificity of Siglec-7 to RCC-associated gangliosides such as DSGb5 (Ito, Handa, et al. 2001). To clarify the functional role of DSGb5 in RCC metastases, we investigated in the present study whether DSGb5 expressed on RCC cells can modulate NK cell cytotoxicity in a Siglec-7-dependent manner. In this report, we discuss the results and the functional implications of RCC metastases.

Results

DSGb5 expression of ACHN cells transfected with siRNA expression plasmid vector for ST6GalNAcVI cDNA

The mRNA level of hST6GalNAcVI in siRNA and mock transfectants was investigated by real-time reverse transcription polymerase chain reaction (RT-PCR), and the relative expression level of hST6GalNAcVI in the siRNA transfectant was about 50% lower than that in the mock transfectant (data not shown). The specificity of siRNA showed more than 50% knockdown effect at the DSGb5 ganglioside level. Monoclonal stable transfectant cells were established by selection with puromycin, and their glycolipid expression patterns did not change after several passages as confirmed by TLC and flow cytometry using anti-DSGb5 mAb.

The flow cytometric analysis demonstrated that DSGb5 expression in the siRNA transfected was greatly reduced to almost half the level in the mock transfectant (Figure 1A). The expression level of MSGb5 was similar in both transfectants. The TLC pattern of gangliosides extracted from the siRNA transfecant showed a reduced DSGb5 expression and a similar expression of MSGb5 compared with the mock transfectant (Figure 1B). DSGb5 and MSGb5 expression was confirmed by TLC immunostaining with mAbs 5F3 (anti-DSGb5) and RM1 (anti-MSGb5) (data not shown).

Binding ability of ACHN cells to Siglec-7-Fc chimera protein

ACHN cells that expressed a high level of DSGb5 showed strong binding ability to Siglec-7. In these experiments, the binding activity of ACHN cells was considerably reduced by pre-incubation with 5F3 (anti-DSGb5 mAb) but not with RM1 (anti-MSGb5 mAb) (Figure 2A). Furthermore, the binding ability to Siglec-7 of ACHN cells transfected with the siRNA vector was markedly weaker than that of mock vector transfectants (Figure 2B).

Therefore, as the binding ability of ACHN cells was attenuated by the anti-DSGb5 mAb 5F3 and reduced by downregulation of DSGb5 with siRNA transfection, the binding ability of ACHN cells to Siglec-7 depended on the expression level of DSGb5 on the cell surface. We consider that DSGb5 expressed on the cell surface of ACHN cells is the key molecule at the point of interaction between RCC cells and NK cells.

Cytotoxic activity of NK cells against RCC cells modulated by DSGb5 expression

There is masking status of Siglec-7 on NK cells within peripheral blood mononuclear cells (PBMCs), as mentioned above (Falco et al. 1999; Nicoll et al. 1999). Without sialidase treatment, the cytotoxic activity of NK cells was almost the same among the two different transfectants, as shown in Figure 3A. When Siglec-7 was unmasked on PBMCs by sialidase treatment, the cytotoxic activity of NK cells against the mock transfectant was significantly inhibited compared with the siRNA transfectant (Figure 3B). These results demonstrate that unmasked Siglec-7 expressed on NK cells can interact with DSGb5 presented on RCC cells, thus decreasing the cytotoxicity of NK cells.

Discussion

Studies of GSLs have suggested that RCC metastasis is correlated with the expression of higher gangliosides with TLC mobility similar to or slower than that of GM2 (Saito et al. 1991). Among these gangliosides, DSGb5 and MSGb5 (which have an extended globo-series core) were identified from RCC tissues, including metastatic RCC tissues (Saito et al. 1994). DSGb5 is derived from MSGb5 with one sialic acid added via ST6GalNAcVI, which is one of the sialyltransferases (Senda et al. 2007). In a previous study, we showed that chemically purified MSGb5 containing α2, 3-linked sialic acid at the terminus does not bind to Siglec-7, but DSGb5 containing α2, 3-linked sialic acid and internally branched α2, 6-linked sialic acid binds to Siglec-7 (Ito, Handa, et al. 2001). In the present study, we confirmed that ACHN cells with high expression levels of DSGb5 bound to Siglec-7-Fc fusion chimera protein and that the binding activities of ACHN cells to Siglec-7 in the siRNA transfectant were less than those in the mock transfectant. In addition, these binding activities were only inhibited by the anti-DSGb5 mAb 5F3 and not by the anti-MSGb5 mAb RM1. These observations showed that Siglec-7 specifically binds to DSGb5 expressed on the surface of RCC cells and that a function of DSGb5 expressed on RCC cells is the ligand for Siglec-7 during cell–cell interactions.

After the binding assay, we performed a cytotoxicity assay using NK and ACHN cells. Because Siglec-7 expressed on NK cells is masked by sialic acid, NK cells cannot bind via Siglec-7 to DSGb5 without sialidase treatment (Razi and Varki 1998; Razi and Varki 1999; Nicoll et al. 2003). We showed that the cytotoxicity of NK cells was almost the same in the siRNA transfectant and the mock transfectant lacking sialidase treatment because of the masked Siglec-7. However, once the NK cells had been treated with sialidase and Siglec-7 was unmasked, the cytotoxicity against ACHN cells with high DSGb5 expression (mock transfectant) was decreased relative to cells with low DSGb5 expression (siRNA transfectant). These findings suggest that the cytotoxicity of NK cells against ACHN cells was inhibited by interaction between Siglec-7 on effector cells (NK cells) and DSGb5 on target cells (ACHN cells).

DSGb5 expressed on RCC cells has been identified in previous studies as a tumor metastasis-related ganglioside. However, the functional role of DSGb5 in RCC metastasis has not yet been clarified. In the present study, we have shown that DSGb5 expressed on RCC cells can decrease the cytotoxicity of NK cells via Siglec-7-dependent mechanisms. DSGb5 is initially expressed in the proximal tubules of a normal kidney, and the amount of DSGb5 in normal tissues is not as remarkable.
The physiological significance of the glycosyl epitope on nonmalignant epithelial cells, which binds to a leukocyte inhibitory receptor Siglec-7, is possibly to protect epithelial cells from unexpected cytotoxic attack by autologous lymphocytes and monocytes (Miyazaki et al. 2004). RCC cells are thought to originate from the proximal tubules of the normal kidney, and in the process of becoming malignant, they amplify the degree of DSGb5 expression and acquire the ability to escape from cytotoxicity of NK cells through an interaction between DSGb5 and Siglec-7. Consequently, cancer cells could create favorable circumstances for their own survival and for metastasis.

Despite demonstrating the interaction between Siglec-7 and DSGb5, we cannot directly conclude that unmasked Siglec-7 expressed on NK cells interacts with DSGb5 presenting on RCC cells; we would need to include a blocking Siglec-7 antibody to prove that this is due to Siglec-7 rather than another effector pathway that binds to DSGb5. Therefore, it is important for us to further investigate the properties (e.g., as another effector and as a ligand for signal transduction) of DSGb5 expressed on RCC cells. We are presently examining the biological activities of RCC cells that express DSGb5. Possible functional roles of DSGb5 in RCC metastases are illustrated in Figure 4. Further understanding of the glycobiological me-
In conclusion, we have suggested a functional role of the ganglioside DSGb5 in RCC metastasis and confirmed that NK cell cytotoxicity against ACHN cells was inhibited by interaction between Siglec-7 on NK cells and DSGb5 on RCC cells. Therefore, the regulation of DSGb5 using glycobiological approaches could have the potential to reduce the malignancy of RCC, and this strategy may lead to the development of a novel therapy for RCC patients.

**Materials and methods**

**Cell lines**

ACHN cells were purchased from Dainipponseiyaku Co. (Tokyo, Japan). These cells were originally derived from malignant pleural effusion of a patient with widespread metastatic RCC. This cell line was maintained in high-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco-Invitrogen, Rockville, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (ICN Biomedicals, OH, USA).

**Monoclonal antibodies**

The following mAbs were established in our laboratory: mouse IgM anti-DSGb5 mAb 5F3 established using ACHN cells (Ito, Saito, et al. 2001) and mouse IgM anti-MSGb5 mAb RM1 (Saito et al. 1994). The ganglioside structures used in the present study and the mAbs defining them are summarized in Table I (Ito, Saito, et al., 2001).

**Flow cytometric analysis**

The expression of gangliosides on the cell surface was analyzed using a FACScan flow cytometer with CellQuest software (BD Biosciences, CA, USA). Cells were incubated with 20 μg/mL mAbs (normal mouse IgM (Syntosome, Munich, Germany),
Table I. Gangliosides present in human renal cell carcinoma and monoclonal antibodies defining them

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>mAb</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSGb5 (monosialosyl globopentaosylceramide)</td>
<td>NeuAcα3Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer</td>
<td>RM1 (1)</td>
</tr>
<tr>
<td>DSGb5 (disialosyl globopentaosylceramide)</td>
<td>ST6GalNAcVI</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NeuAcα3Galβ3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GalNAcβ3Galα4Galβ4Glcβ1Cer</td>
<td>5F3 (2)</td>
</tr>
<tr>
<td></td>
<td>NeuAcα6</td>
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5F3, and RM1) for 1 h on ice. After washing with phosphate-buffered saline (PBS) + 1% bovine serum albumin (BSA), cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat antibody directed to mouse IgG and IgM (Jackson ImmunoResearch Laboratories, PA, USA) for 30 min on ice, washed, and then analyzed by flow cytometry.

**Glycolipid extraction and thin-layer chromatography**

GSLs were extracted from ACHN cells, as described previously (Ito, Levery, et al. 2001; Satoh et al. 2001). Briefly, 2–5 × 10⁷ cells (pelleted by centrifugation) were extracted twice with 2 mL of isopropanol/hexane/water (55:25:20 v/v/v) (Ito, Levery, et al. 2001; Satoh et al. 2001). Extracts were evaporated to dryness under a nitrogen stream, and phospholipids in the residue were dissolved in 2 mL of 0.1 M NaOH in methanol (40°C, 2 h), neutralized with 200 μL of 1 N HCl, and then shaken repeatedly with 2 mL of hexane to eliminate fatty acids. GSLs in the lower layer were further purified and desalted using a Bond Elut C18 solid-phase extraction cartridge (Varian, CA, USA) (Tsuboi et al. 1991). Total eluates were dissolved in chloroform/methanol (C/M) 2:1 and then subjected to TLC analysis (Tsuboi et al. 1991; Ito, Handa, et al. 2001). Aliquots of GSL fractions placed on the TLC plate for analysis were based on equal wet cell weight (10 mg). For example, the wet weight of 3 × 10⁷ pelleted ACHN cells is usually 200 mg. The GSL fraction derived from these cells is dissolved in 100 μL of C/M 2:1; a 15-μL aliquot (equivalent to 10 mg wet cell weight) is spotted onto a TLC plate, developed in C/M/0.5% aqueous CaCl₂ (50:40:10 v/v/v), and then visualized by spraying with 0.5% orcinol in 2N sulfuric acid.

**Establishment of Siglec-7-Fc fusion chimera protein**

The pcDM8 vector containing the full-length Siglec-7 sequence (as described previously) was digested with Hind III and Stu I to purify the fragment of the extracellular domain of Siglec-7 (Ito, Handa, et al. 2001) and ligated to the Sma I and Hind III sites of pBluescript vector to produce pBluescript with a Siglec-7 insert. The pcDNA3 vector was digested with BamH I and Xba I and ligated to the BamH I and Xba I sites of HICOSig (a generous gift from Dr. Ledbetter, Pacific Northwest Research Institute, Seattle, WA, USA) to produce pcDNA3 with the human IgG Fc domain. pBluescript-Siglec-7 was digested with Hind III and Bam I and ligated to the Hind III and Bam I sites of pcDNA3-IgFc to produce the pcDNA3 vector containing a fusion of the Siglec-7 extracellular domain and human IgG Fc domain.

COS7 cells were transfected with pcDNA3-Siglec-7-Fc and cultured for 2 days in DMEM with 10% FBS. The supernatants containing Siglec-7-Fc were collected, and the expression of Siglec-7-Fc was determined by enzyme-linked immunosorbent assay using anti-human Siglec-7 antibody (R&D systems, MN, USA).

**Binding studies of ACHN cells to recombinant Siglec-7-Fc chimera protein**

ACHN cells were detached with 0.05% trypsin and 0.53 mM EDTA, washed with 10% FBS/DMEM, suspended in 1 mL of 10% FBS/DMEM with 10 μM calcein-AM (Invitrogen, Rockville, MD, USA) at a concentration of 5 × 10⁶/mL, incubated for 45 min at 37°C, and then washed with DMEM. Fluorescence-labeled cells were suspended in 1% BSA/DMEM at a concentration of 5 × 10⁵/mL.

A 100-μL aliquot of goat anti-human IgG (Fc-fragment specific) (Jackson ImmunoResearch Laboratories) was added to each well of a 96-well flat bottom polystyrene plate (Falcon 3915, Becton Dickinson, NJ, USA), incubated overnight at 4°C, and washed in PBS. Culture supernatant (100 μL) containing Siglec-7-Fc chimera protein was added to each well and incubated for 4 h at room temperature. Each well was washed with PBS and blocked with 3% BSA/PBS for 1 h at room temperature and then washed three times with PBS. Thereafter, 200 μL of 1% BSA/DMEM and 100 μL of calcein-AM-labeled cell suspension (5 × 10⁴ cells/well) were added. The plate was incubated and shielded from light for 1 h at room temperature and then washed using the following procedure known as gravity wash, as described previously (Handa et al. 1997; Ito, Handa, et al. 2001). Briefly, the plate was immersed in PBS (plus 0.9 mM CaCl₂ and 0.5 mM MgCl₂) in a large container and floated upside-down for 10 min to allow non-adherent cells to fall out of the wells. The plate was then turned right-side up and removed from the container. PBS (200 μL) was removed from each well, and the number
of remaining cells adhering to Siglec-7-Fc-coated wells was calculated based on the fluorescence of each well measured at 535 nm (excitation at 485 nm) on a Fluoroskan Ascent Plate Reader (LabSystem, Dainipponseiyaku). For antibody-blocking experiments, ACHN cells were pre-incubated with each mAb (5F3 or RM1) for 30 min at 37°C and washed with 1% BSA/DMEM prior to the binding studies.

Establishment of stable transfecant of siRNA expression plasmid vector for ST6GalNAcVI

hST6GalNAcVI belongs to a family of sialyltransferases that modify proteins and ceramides on the cell surface to alter cell–cell or cell–extracellular matrix interactions; the Gene Bank Accession No. of this sialyltransferase is M013443.3 (Gene ID 30815) (Tsushida et al. 2003). siRNAs targeting hST6GalNAcVI and control scrambled siRNAs were obtained from B-Bridge International, Inc. (CA, USA). The sequence for targeting hST6GalNAcVI siRNA was CCAAUGAGGUCUUCANUUA beginning at nucleotide 191 of the ST6GalNAcVI open reading frame sequence, and that for the scrambled siRNA control was UAUCGUUAACUAGAUCCCG. Transfection into ACHN cells was performed in 24-well plates with Lipofectamine 2000 (Invitrogen) (Senda et al. 2007). At 72–84 h after transfection, knockdown efficiency was examined between two transfecant cells. The 21-nt sequences were purchased from B-Bridge (Tokyo, Japan, and the specificity of these sequences to transfect to ACHN cells was confirmed with Lipofectamine 2000. The siRNA expression plasmid vector-establishing protocol developed by iGENE (http://igene-therapeutics.co.jp/) (Sapporo, Japan) was purchased from TAKARA Bio (Tokyo, Japan), and a mock plasmid vector was obtained. For plasmid DNA, pBAsi-hU6 Pur DNA was used for siRNA expression and the mock vector.

ACHN cells were plated at a density of 5 × 10³ per 35-mm diameter dish. After 1 day, ACHN cells were transfected with 200 pmol siRNA expression plasmid DNA using Lipofectamine 2000, according to the manufacturer’s protocol. The medium was replaced with fresh medium 6 h after transfection and incubated with 5 ng/mL puromycin in DMEM + 10% FBS to establish puromycin-resistant colonies.

The gene silencing effect was analyzed more than 7 days after selecting colonies by RT-PCR using LightCycler (Roche, Germany) and flow cytometry. The mock plasmid DNA transfecant was confirmed as above.

Leukocyte isolation and NK cell cytotoxicity assay with or without sialidase treatment

PBMCs were isolated from the blood of healthy donors by Ficoll-Paque (Amersham Biosciences, NJ, USA) density gradient centrifugation (Mangan et al. 1984). For the cytotoxicity assays, monocytes were removed by adherence to tissue culture dishes for 1 h at 37°C, and the nonadherent PBMCs containing lymphocytes and NK cells were washed with Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco-Invitrogen) containing 10% FBS. Cells were suspended at 5 × 10⁵/mL in 0.25% BSA/RPMI-1640 and incubated in the presence or absence of 0.1 U/mL Vibrio cholerae sialidase (Calbiochem, La Jolla, CA, USA) for 1.5 h at 37°C, followed by extensive washing in 10% FBS/RPMI-1640 (Razi and Varki 1998, 1999; Nicoll et al. 2003). The presence of NK cells in peripheral blood lymphocytes (PBLs) was evaluated by fluorescence-activated cell sorting (FACS) analysis (see above) using anti-CD56-FITC Ab (Becton Dickinson Biosciences).

ACHN target cells (1 × 10⁵/mL) were labeled for 30 min by incubating with 10 μM fluorescent dye calcein-AM (Invitrogen) in 10% FBS/DMEM at 37°C. The cells were washed and suspended in 10% FBS/RPMI at a concentration of 5 × 10⁴ cells/mL. NK cytotoxic activity against ACHN cells was measured using the Calcein-AM-releasing assay, as described previously (Imai et al. 1995). Briefly, 100 μL of ACHN target cells was added to each well of a V-bottom 96-well microtitre plate and mixed with varying ratios of PBLs. Spontaneous release of calcein-AM was determined by incubation of the ACHN cells in the 10% FBS/RPMI medium alone. The plate was centrifuged at 3000 rpm for 5 min and incubated for 6 h at 37°C, and then 100 μL of 2% Triton X-100 in RPMI + 10% FBS was added to each well. Samples (80 μL) of the supernatant from each well were analyzed using a FluoroSkant plate reader. The percentage of specific cell lysis was calculated using the formula: % of specific lysis = (experimental release – spontaneous release)/(maximum release – spontaneous release) (Imai et al. 1995; Neri et al. 2001).

Statistical analysis

Values represent the means ± S.D. of at least triplicate readings, and the Student’s t-test was used for data analysis.

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Conflict of interest statement

None declared.

Abbreviations


BSA, bovine serum albumin; C/M, chloroform/methanol; DMEM, Dulbecco’s modified Eagle’s medium; DSGb5, disialosyl globobiosylceramide (V³NeuAcIV³NeuAcGb5); DSLc4, disialosyl Lc4 (IV³NeuAcIII6NeuAcLc4); FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GSL, glycosphingolipid; mAb, monoclonal antibody; MSGb5, monosialosyl globobiosylceramide (V⁵NeuAcGb5); NK, natural killer; PBL, peripheral blood lymphocyte; PBMCs, peripheral blood mononuclear cells; PBS, phosphate-buffered saline (2.67 mM KCl, 1.47 mM K₂HPO₄, 138 mM NaCl, 8.1 mM Na₃HPO₄-7H₂O); RCC, renal cell carcinoma; RTPCR, reverse transcription polymerase chain reaction; Siglec,
sialic acid/immunoglobulin/lectin (Crocker PR et al., Glycobiology 8(2): v, 1998); TLC, thin-layer chromatography.

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