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Binding specificity of siglec7 to disialogangliosides of renal cell carcinoma: possible role of disialogangliosides in tumor progression☆

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Abstract Previous studies indicate that expression of higher gangliosides in renal cell carcinoma (RCC) is correlated with metastatic potential, particularly in the lung. Out of five major gangliosides in RCC, three disialogangliosides (disialogalactosylgloboside, IV^{3}NeuAcIII^{6}NeuAcLc$_4$, and IV^{4}GalNAcIV^{3}NeuAcIII^{6}NeuAcLc$_4$) bind strongly to siglec7, which is expressed highly in monocytes and natural killer cells. Out of other gangliosides tested, 2→6 sialylparagloboside, GD3, GD2, and GT1b, but not other lacto- or ganglio-series gangliosides, showed clear binding to siglec7. In view of preferential metastasis of RCC to the lung, and binding of RCC cell line TOS-1 to lung tissue sections as shown in our previous study, we examined expression of siglec7 in the lung. siglec7 is expressed highly in resident blood cells, but not in parenchymatous cells. TOS-1 cells aggregate together strongly through adhesion with peripheral blood mononuclear cells to form large clumps. This suggests the possibility that such aggregates may form embolisms of microvasculature, particularly in the lung, which initiate metastasis. Other possible roles of higher gangliosides in RCC in promoting metastasis and tumor progression are discussed.

Key words: Renal cell carcinoma; Metastasis; Ganglioside; Siglec7; Adhesion; TOS-1 cell

1. Introduction

Renal cell carcinoma (RCC) is highly malignant, and distant metastasis is often already present at the time of first diagnosis. A study of glycosphingolipids (GSLs) expressed in RCC primary and metastatic lesions indicated a correlation of the presence of thin-layer chromatography (TLC) slow-migrating gangliosides with metastasis [1,2]. Among these gangliosides, monosialogalactosylgloboside (MSGG) and disialogalactosylgloboside (DSGG) were previously identified from RCC tissue extract [3]. We recently identified two additional gangliosides from the metastatic RCC cell line TOS-1, termed respectively IV^{3}NeuAcIII^{6}NeuAcLc$_4$ (DSLc$_4$) and IV^{4}GalNAcIV^{3}NeuAcIII^{6}NeuAcLc$_4$ (GalNAcDSLc$_4$) [4] (for structures and mAbs defining them, see Table 1). Expression of GalNAcDSLc$_4$ and MSGG, defined respectively by mAbs RM2 and RM1 in primary RCC lesion, was correlated with higher incidences of distant metastasis to the lymph node, lung, and other organs [5][1].

This correlation observed in RCC is strikingly different from the situation in some other types of cancer, in which sialyl-Lewis^x (SLe^x) and SLe^a show preferential metastasis, possibly through binding of these epitopes to E-selectin expressed on activated endothelial cells (for review see [6,7]). However, SLe^x, SLe^a, and their analogues are minimally expressed in RCC and have no association with RCC metastasis [1]. There are several possibilities for ganglioside involvement in the promotion of metastasis (see Section 4). Sialic acid binding proteins (siglecs (sialic acid/immunoglobulin/lectin)) [8] may mediate binding of sialoglyco conjugates expressed on tumor cells to certain target cells which express siglecs, although sialoglycoproteins are the major targets of siglecs, and little attention has been paid to gangliosides. One exception is myelin-associated glycoprotein, i.e. siglec4, which mediates myelin–neuron interaction by binding of ganglio-series gangliosides [9]. A possible role of siglecs in mediating tumor cell adhesion through sialoglyco conjugates expressed on tumor cells has been considered. Of particular interest is siglec7, which is expressed in natural killer (NK) cells and able to affect NK activity through binding [10,11]. We therefore studied the binding specificity of siglec7 to various RCC-asso-

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Abbreviations: BSA, bovine serum albumin; DSLc$_4$, IV^{3}NeuAcIII^{6}NeuAcLc$_4$; DSGG, disialogalactosylgloboside (V^{3}NeuAcIV^{6}NeuAcGb5); GalNAcDSLc$_4$, IV^{4}GalNAcIV^{3}NeuAcIII^{6}NeuAcLc$_4$; GSL, glycosphingolipid; MSGG, monosialogalactosylgloboside (V^{3}NeuAcGb5); PBMC, peripheral blood mononuclear cells; PBS, 2.67 mM KCl, 1.47 mM K_2HPO_4, 138 mM NaCl, 8.1 mM $Na_2HPO_4{\cdot}7H_2O$; RCC, renal cell carcinoma; siglec, sialic acid/immunoglobulin/lectin (Crocker et al., Glycobiology 8 (1998) v); 2→3 SnLc$_6$, 2→3 sialylnorhexaosylceramide (VI^{3}NeuAcnLc$_6$); 2→3 SPG, 2→3 sialylparagloboside (IV^{3}NeuAcnLc$_4$); 2→6 SPG, 2→6 sialylparagloboside (IV^{6}NeuAcnLc$_4$); SPG, sialylparagloboside; TLC, thin-layer chromatography; SLe, sialyl-Lewis; NK, natural killer; Glycosphingolipids are abbreviated as recommended by IUPAC-IUB (J. Biol. Chem. 257 (1982) 3347–3351, 262 (1987) 13–18). Gangliosides are abbreviated according to Svennerholm (J. Lipid Res. 5 (1964) 145–155)

[1] Further supplemental data on reactivity of RCC tissues with various antibodies, including new mAb 5F3 directed to DSGG, as correlated with distant metastasis, were presented by Akihiro Ito (PhD Thesis, Tohoku University School of Medicine, Japan, 1997).

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Table 1
Major gangliosides present in human RCC, and monoclonal antibodies defining them

Name	Structure	mAb
GM2	$\begin{matrix}\text{NeuAc}\alpha3\\ \text{GalNAc}\beta4\end{matrix}Gal\beta$4Glc$\beta$1Cer	MK1-8 (a)
MSGG (monosialogalactosyl-globoside)	NeuAcα3Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer	RM1 (b)
DSGG (disialogalactosyl-globoside)	$\begin{matrix}\text{NeuAc}\alpha3\text{Gal}\beta3\\ \text{NeuAc}\alpha6\end{matrix}$GalNAc$\beta$3Gal$\alpha$4Gal$\beta$4Glc$\beta$1Cer	5F3 (c)
$DSLc_4$ (disialyl lactotetraosyl-ceramide)	$\begin{matrix}\text{NeuAc}\alpha3\text{Gal}\beta3\\ \text{NeuAc}\alpha6\end{matrix}$GlcNAc$\beta$3Gal$\beta$4Glc$\beta$1Cer	FH9 (d)
$GalNAcDSLc_4$ (disialyl GalNAc lactotetraosylceramide)	$\begin{matrix}\text{NeuAc}\alpha3\\ \text{GalNAc}\beta4\end{matrix}\begin{matrix}\text{Gal}\beta3\\ \text{NeuAc}\alpha6\end{matrix}$GlcNAc$\beta$3Gal$\beta$4Glc$\beta$1Cer	RM2 (e)

(a) Donated by Reiji Kannagi [31]. (b) Saito et al. [3]. (c) Footnote 1. (d) Fukushi et al. [32]. (e) Ito et al. [4].

ciated gangliosides, as above, in comparison to other gangliosides. Results and their functional implications are discussed in this paper.

2. Materials and methods

2.1. Glycolipids

$GalNacDSLc_4$ and $DSLc_4$ were prepared from the TOS-1 cell line, derived from back metastatic lesion of an RCC patient [12]. DSGG and MSGG were prepared from ACHN cells (human RCC cell line). 2→6 sialylparagloboside (SPG) was prepared from human colonic adenocarcinoma, 2→3 SPG from human placenta, 2→3 sialylnorhexaosylceramide (2→3 $SnLc_6$) from human erythrocytes, and SLe^a from pancreatic cancer tissue. SLe^x was prepared by fucosylation of SPG. GM1, GM2, GD1a, GD1b, GD3, and GT1b were purchased from Matreya, Inc. (Pleasant Gap, PA, USA), and GD2 from Sigma (St. Louis, MO, USA).

2.2. Monoclonal antibodies

Anti-siglec7 mAb S7.7a was donated by Dr. P.R. Crocker [10]. Anti-P-selectin mAb P5A [13] and anti-type 2 chain H mAb BE2 [14] were established in our laboratory. Other mAbs directed to gangliosides and their sources are listed in Table 1.

2.3. Construction of siglec7 expression plasmid

Siglec7 cDNA was cloned in two steps. First, most of the encoding region including the carboxy-terminus was cloned by PCR using a commercially available human lung cDNA library as the template and primers derived from overlapping EST clones (5′ primer GACGTCTAGATCCCCGGGTGACCGTGCAAGAGGGCATGT derived from EST clone AA344713 and 3′ primer GGGGAAAGG-GAGAGTTTGGTCATCAGGCATGCAT derived from EST clone T48551).

The remaining 5′ portion was cloned by RT-PCR using RNA from human NK cells (purified from circulating blood using anti-CD56 conjugated to magnetic beads; Dynal Inc., Lake Success, NY, USA) as the template. The RT reaction was primed with a siglec7 gene-specific primer (CATACGAAAGAAGTAT), Superscript II (Life Technologies, Grand Island, NY, USA) and conditions suggested by the manufacturer. PCR used published sequences to derive the 5′ (GCCAAGCTTAACCCCAGATATGCTGCTG) and 3′ (TCCCGAGTTTCCTCCTGCAC) primers.

The C-terminal encoding fragment (cut with *Eae*I and *Xho*I), the N-terminal encoding fragment (cut with *Hin*dIII and *Xho*I) and the pCDM8 expression vector (cut with *Hin*dIII and *Xho*I) were ligated and a clone containing the full length siglec7 sequence was selected and confirmed by sequencing.

2.4. Transient transfection of full-length siglec7

COS-7 cells were plated at a density of 4×10^5/60-mm culture dish, incubated overnight, transiently transfected with plasmid cDNA of pCDM8-siglec7 or vector pCDM8 using FuGENE6 transfection reagent (Roche, Indianapolis, IN, USA), and incubated for 48 h prior to the binding assay. Expression of siglec7 in COS-7 cells was determined by binding of human erythrocytes, as shown in Fig. 1 and explained in its legend.

2.5. Binding specificity of siglec7-transfected COS-7 cells to various gangliosides

Cells were detached with 0.05% trypsin and 0.53 mM EDTA, washed with Dulbecco's modified Eagle's medium (DMEM), suspended in 1 ml DMEM with 5 μM fluorescent dye (calcein-AM, Molecular Probe, Eugene, OR, USA) at a concentration of 5×10^6/ml, incubated for 45 min at 37°C, washed with DMEM, and fluorescence-labeled cells were suspended in 1% bovine serum albumin (BSA) in DMEM at a concentration of 5×10^5/ml.

Gangliosides were dissolved in ethanol (5 nmol/ml). A 50 μl aliquot and its serially diluted solution were added to each well of a 96-well flat bottom polystyrene plate (Falcon 3915, Becton Dickinson, NJ, USA), dried at 37°C, and washed in PBS (2.67 mM KCl, 1.47 mM K_2HPO_4, 138 mM NaCl, 8.1 mM Na_2HPO_4–$7H_2O$). Each well was blocked with 1% BSA in DMEM for 1 h at 37°C, washed in PBS three times, and added with 200 μl of 1% BSA in DMEM and 100 μl

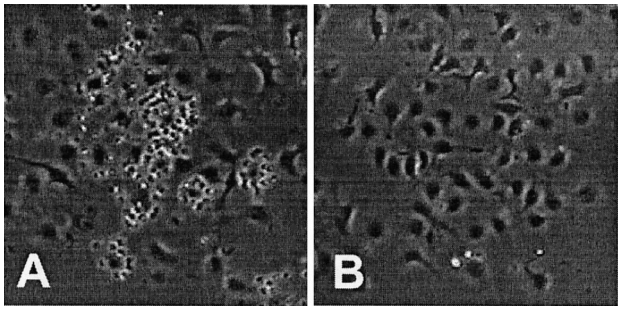

Fig. 1. Binding of human erythrocytes to siglec7-transfected COS-7 cells. 48 h after transfection, COS-7 cells were detached, plated on 48-well plates at a concentration of 1×10^5/well, and incubated for 4 h. 0.25% erythrocytes (v/v) in 1% BSA/DMEM (pH 7.4) were added to each well, and incubated for 30 min at 37°C. Each well was washed with medium three times, and cells were observed by microscopy. A: Siglec7-transfected COS-7 cells. Bar, 100 μm. B: Control COS-7 cells.

of cell suspension (5×10^4 cells per well). The plate was incubated with shielding light for 1 h at room temperature and then washed by the following special procedure [15]. The plate was immersed in PBS(+) (plus 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$) 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 rightside-up and removed from the container. 200 μl PBS of each well was removed and remaining cells were lysed with 100 μl 1% Triton X-100. A 100 μl aliquot of each well was transferred to a fresh 96-well plate, and the number of cells adhered to ganglioside-coated wells was calculated based on fluorescence of each well quantified at 530 nm (excitation at 485 nm) on a CytoFluor Multiwell Plate-Reader 4000 (PerSeptive BioSystem, Framingham, MA, USA).

2.6. Immunohistochemical staining of normal human lung sections

Surgically resected normal human lung tissues were donated by the Department of Thoracic Surgery, Institute of Development, Aging, and Cancer, School of Medicine, Tohoku University, Japan. Cryostat sections (8 μm) were mounted on microslides, fixed in cold acetone, blocked with 1% defatted milk in PBS, and incubated with primary antibodies overnight at 4°C. Endogenous peroxidase was blocked with 0.3% H_2O_2 in PBS for 5 min. Sections were incubated with biotinylated secondary antibody for 1 h, incubated with Vector avidin–biotin solution for 1 h, and stained with 0.05% 3,3′-diaminobenzidine and 0.01% H_2O_2 in 0.05 M Tris–HCl.

2.7. Aggregation of TOS-1 cells with peripheral blood mononuclear cells (PBMC)

TOS-1 cells were detached with 0.05% trypsin and 0.53 mM EDTA, washed, and suspended in 1% BSA in DMEM. PBMC were isolated from heparinized blood of healthy donors by sedimentation on Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, NJ, USA) gradients and labeled with fluorescent Calcein-AM as described in Section 2.5. Labeling of lymphocytes and leukocytes by calcein-AM does not alter cellular functions, including cell binding activity [16]. TOS-1 cells (5×10^5/100 μl) and PBMC (2×10^6/100 μl) were mixed, incubated for 30 min at 37°C, and observed under fluorescence microscopy.

3. Results

Three disialogangliosides expressed highly in RCC, i.e. DSGG, $DSLc_4$, and $GalNAcDSLc_4$ (for structures see Table 1), showed strong binding to siglec7 expressed on COS-7 cells transfected with the siglec7 gene (Fig. 2A,B). Clear cell binding was observed for 8–16 pmol/well of these three gangliosides. In contrast, clear antibody binding was observed for 0.8–1.6 pmol/well of the same gangliosides. MSGG and GM2, both expressed highly in RCC, did not show clear binding to siglec7 (Fig. 2A,D). In contrast, various disialogangliosides with ganglio-series structure, i.e. GD3, GT1b, GD2, showed strong binding (Fig. 2D). GD1a, GD1b, GM1, SLe^x, and SLe^a showed no binding (Fig. 2B,D). Among type 2 lacto-series gangliosides, 2→6 SPG showed strong binding, whereas 2→3 SPG and 2→3 $SnLc_6$ showed no binding (Fig. 2C). In these experiments, binding activity and specificity of various gangliosides were highly reproducible when using COS-7 cells with comparable degree of siglec7 expression.

Because lung tissue is the most frequent site of RCC metastasis, we previously studied binding of RCC-derived TOS-1 cells to various frozen tissue sections [17] by the Stamper–Woodruff method [18] and found that TOS-1 cells showed preferential binding to the perialveolar area of lung tissue. In addition, the siglec7 messenger was reported to be expressed in the lung [10,19], and we confirmed this observation. A possibility is therefore suggested that TOS-1 cell binding to lung tissue is due to expression of siglec7 in the normal lung.

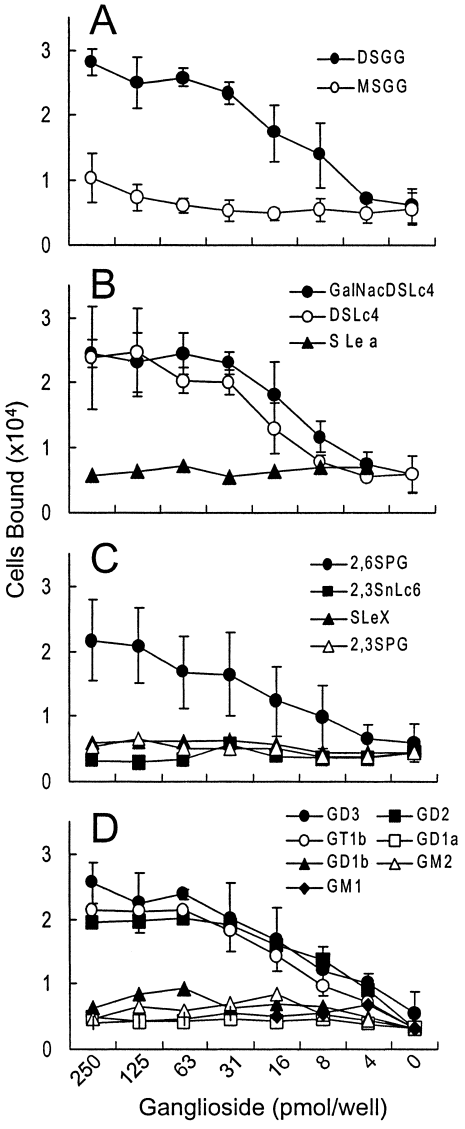

Fig. 2. Binding specificity of siglec7-transfected COS-7 cells to various gangliosides. 48 h after transfection, detached COS-7 cells were labeled with calcein-AM, and suspended in 1% BSA in DMEM at a concentration of 5×10^5/ml. Serially diluted gangliosides were coated to 96-well flat bottom polystyrene plates (approximate quantity in pmol per well is shown on abscissa). After blocking with 1% BSA in DMEM, 100 μl of cell suspension (5×10^4 cells per well) was added, incubated for 1 h at room temperature with shielding light, and the plate was washed as described in the text. Adherent cells were lysed with 1% Triton X-100, and fluorescence of each well was quantified at 530 nm (excitation at 485 nm). Each point represents mean value of three separate experiments using COS-7 cells with similar degrees of siglec7 expression. Vertical bar, standard variation. A: Globo-series gangliosides. B: Lacto-series type 1 chain gangliosides. C: Neolacto-series type 2 chain gangliosides. D: Ganglio-series gangliosides.

However, lung tissue contains numerous monocytes, macrophages, and other types of leukocytes, which are known to express high levels of various siglecs, including siglec7 [10]. We confirmed that anti-siglec7 mAb S7.7a strongly stained blood cells. We therefore studied expression of siglec7 in parenchymatous cells of normal lung tissue. mAb S7.7a did not immunostain endothelial cells, bronchioli, or other parenchymatous cells (Fig. 3, panel 1C), whereas positive staining was observed

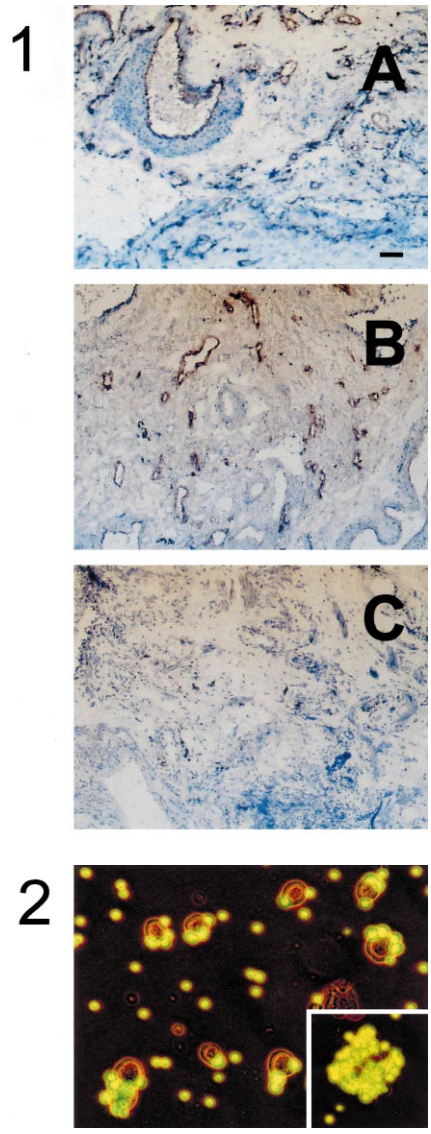

Fig. 3. Immunohistochemical staining of siglec7 expressed in normal human lung frozen sections, and typical aggregates of RCC-derived TOS-1 cells with PBMC. 1A: Staining with anti-type 2 chain H mAb BE2; indicates mucous membrane (bronchiolus). Bar, 100 μm. 1B: Staining with anti-P-selectin P5A; indicates endothelial cells. 1C: Staining with anti-siglec7 S7.7a. No staining is seen in parenchymatous tissue. Areas with presence of resident blood cells are omitted. 2: TOS-1 cells (large cells) and PBMC labeled with calcein-AM (small green fluorescent cells) were mixed, and incubated for 30 min at 37°C. Cells were observed under fluorescence microscopy. Inset, one of the typical large clumps (tumor cell aggregate surrounded by PBMC) occasionally observed.

for control markers (P-selectin for endothelial cells; H-antigen for bronchioli) (Fig. 3, panels 1A,B). When TOS-1 cells were incubated with fluorescence-labeled PBMC, as described in Section 2, they aggregated together to form clumps (Fig. 3, panel 2) or larger mixed clumps in which tumor cell aggregates are covered by PBMC (inset of Fig. 3, panel 2).

4. Discussion

Previous studies suggested that RCC metastasis is correlated with expression of disialogangliosides having an extended globo-series core, DSGG [3], or lacto type 1 chain core, $DSLc_4$ or $GalNAcDSLc_4$ [4]. We demonstrate in this paper that: (i) these gangliosides highly expressed in RCC bind strongly to siglec7 expressed on COS-7 cells, (ii) RCC cell line TOS-1 binds to PBMC and other types of blood cells expressing siglec7, leading to aggregation of TOS-1 cells to form large clumps under physiological conditions, (iii) siglec7 expression in lung tissue is due to resident blood cells, whereas siglec7 is absent in parenchymatous lung tissue including endothelial cells of vasculature (marked by P-selectin), bronchioli (marked by H-antigen), and connective tissues. Siglec7 messengers are expressed in various organs including the lung [10] and we confirmed this finding. This is ascribable to the presence of resident blood cells, as described previously [19].

These observations suggest the possibility that RCC metastasis may be mediated by tumor cell aggregation with PBMC and other types of blood cells, and that such clumps may cause embolisms of microvasculature, particularly in the lung. This mechanism may occur in various types of tumor cells besides RCC, mediated by other siglecs besides siglec7. In normal blood (not heparinized blood used in experiments) tumor cell aggregates may include platelets which are activated by tumor cells to release a factor or factors. The factors in turn activate endothelial cells to elicit ICAMs, VCAMs, or E- or P-selectin, which initiate tumor cell adhesion or invasion [20,21]. However, in the case of RCC metastasis, the role of SLe^x and SLe^a through their binding to selectin is negligible [17] in contrast to many other types of tumors [6,7].

A few other functional roles of gangliosides in tumor progression can be considered: (i) tumor cell binding to endothelial cells is mediated by specific carbohydrates, either by interaction of SLe^x, SLe^a, or myeloglycan-type with E-selectin

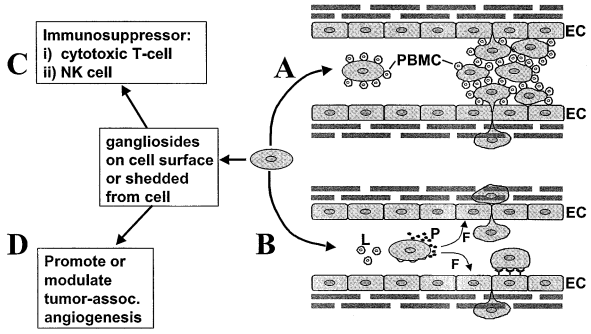

Fig. 4. Possible functional roles of gangliosides in tumor cell metastasis/invasion. Four categories of processes are illustrated. A: Processes based on tumor cell clumping through interaction of tumor cells with PBMC or other blood cells, leading to microembolism. Clumping in normal blood in vivo may include platelets. Both platelets and PBMC, when activated, release factors that activate endothelial cells (EC) and promote tumor cell migration to initiate metastasis (this paper). B: Processes based on tumor cell adhesion to EC without clumping or microembolism. Activation of platelets (P) or lymphocytes (L) induces factors (F) to activate EC [20,21], similar to process A. However, in this case, tumor cell adhesion to EC is based on selectin [6,7,15], integrin–tetraspanin complex [23–25] that binds to ICAM or VCAM, or carbohydrate–carbohydrate interaction [22]. Cell adhesion/motility controlled by integrin complexed with tetraspanin (CD9 or CD82) is strongly modulated by gangliosides [25]. C: Processes based on ganglioside-dependent suppression of immunocytes. Gangliosides on the cell surface, or shed from cells, suppress (i) cytotoxic T-cells [28,29] or (ii) NK cells [11]. D: Processes based on modulation of tumor-associated angiogenesis, e.g. GD3 or GM2 promotes angiogenesis [26,27]. A possible mechanism is effect of these gangliosides on vascular endothelial growth factor receptor function.

[15], or by binding of specific gangliosides highly expressed in tumor cells to endothelial cell GSLs through carbohydrate–carbohydrate interaction, a typical example being the interaction of GM3 (highly expressed on B16 melanoma cells) with LacCer or Gb4 (expressed on endothelial cells) [22], (ii) tumor-associated GSLs modulate adhesion receptors (e.g. integrins) or tetraspanin, to enhance tumor cell adhesion and motility [23–25], (iii) certain gangliosides such as GM2 or GD3 promote tumor-associated angiogenesis [26,27], (iv) tumor-associated gangliosides act as immunosuppressors, as typically observed for suppression of cytotoxic T-cells [28,29], and possible inhibition of NK cells by binding of tumor-associated gangliosides to siglec7 [11]. Possible functional roles of gangliosides in tumor cell metastasis/invasion are illustrated in Fig. 4 and its legend.

Siglecs were discovered originally as 'sialoadhesin' by Crocker and associates. The number of known members of the 'siglec family' has increased greatly through cloning based on the original sequence. They are accordingly expressed in various types of blood cells, including NK cells. Some siglecs have been shown to bind preferentially to sialyl 2→6 Gal, as originally observed for CD22 (siglec2) [30]. Similar preferential binding to sialyl 2→6 Gal was found for siglec7 [19]. Binding of 2→6 SPG but not 2→3 SPG to siglec7, as indicated in the present study, confirmed these previous results. Interestingly, $DSLc_4$, $GalNAcDSLc_4$, and DSGG, which bind strongly to siglec7 and are expressed highly in RCC, are characterized by having sialyl 2→6 GlcNAc or sialyl 2→6 GalNAc. GD3, GD2, and GT1b are expressed highly in numerous types of tumors [6] and are capable of binding to siglec7 (as shown in the present study). Therefore, these disialo epitopes may promote metastasis through a mechanism involving microembolisms, similar to that discussed above, or by other possible mechanisms as illustrated in Fig. 4.

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