Binding of a Sialic Acid-recognitiong Lectin Siglec-9 Modulates Adhesion Dynamics of Cancer Cells via Calpain-mediated Protein Degradation*§

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Background: Siglec-9 binds siaiylglycoconjugates on target cells probably causing signals in both sides of cells.

Results: Co-culture with Siglec-9-expressing U937 caused degradation of focal adhesion kinase and related proteins in an astrocytoma cell line.

Conclusion: Interaction between Siglec-9 and its counterreceptor triggered activation signals in cancer cells via calpain.

Significance: Cancer cells may utilize siaiylglycoconjugates recognized by Siglec-9 to escape from immunosurveillance.

Although regulatory mechanisms for immune cells with inhibitory signals via immunoreceptor tyrosine-based inhibitory motifs are well known, signals transduced via interaction between Siglec and sialyl compounds on their counterreceptors to target cells have not been reported to date. In this study, we found that an astrocytoma cell line, AS, showed detachment from culture plates when co-cultured with Siglec-9-expressing cells and/or soluble Siglec-9. Moreover, detached AS cells regrew as co-cultured cells with Siglec-9-deficient cells. They also showed increased motility and invasiveness upon Siglec-9 binding. In immunoblotting, rapid degradation of focal adhesion kinase (FAK) and related signaling molecules such as Akt, paxillin, and p130Cas was observed immediately after the co-culture. Despite degradation of these molecules, increased p-Akt was found at the front region of the cytoplasm, probably reflecting increased cell motility. Calpain was considered to be a responsible protease for the protein degradation by the inhibition experiments. These results suggest that protein degradation of FAK and related molecules was induced by Siglec-9 binding to its counterreceptors via siaiylglycoconjugates, leading to the modulation of adhesion kinetics of cancer cells. Thus, this might be a mechanism by which cancer cells utilize Siglec-9-derived signals to escape from immunosurveillance.

Sialic acids are present as terminal monosaccharides linked to cell surface glycoconjugates and play a role in the complexity of physiological and pathological events (1). They are often a part of recognition sites for extrinsic pathogens (2), but they also play important roles by interacting with many intrinsic molecules, endogenous lectins. In particular, sialic acid-binding immunoglobulin-like lectins (Siglecs)2 are unique endogenous lectins recognizing sialic acid-containing carbohydrates on glycoproteins and glycosphingolipids (3). There are currently 14 known Siglecs in humans and 9 in mice, and the majority of them are expressed on immune cells except Siglec-4 (3). One of the intriguing findings about Siglecs is that many of them have intracellular domains containing immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and exert as inhibitory receptors to control the immune cells negatively (3). Siglecs have been thought to regulate the functions of innate immune cells and adaptive immune systems based on glycan recognition. In particular, Siglec-7-mediated attenuation of natural killer cell and dendritic cell functions has been reported as an example of escape mechanisms of cancer cells from immune surveillance (4). Siglec-9 was isolated as a gene highly homologous to Siglec-7 (5). It is expressed in monocytes and granulocytes. Although inhibitory functions of Siglec-9 in immune cells via ITIM motifs have been reported in some experimental systems (6), the outcome of the interaction between Siglecs and their sialylated ligands inside cells expressing sialyl compounds has scarcely been studied to date.

During the analysis of effects of interaction between Siglec-9 and its counterligands on cancer cells, we found marked degradation of focal adhesion kinase (FAK) in cancer cells and cell detachment from plates, suggesting apoptosis (7). Consequently, we have obtained interesting findings, suggesting novel implication of Siglec-9-mediated signaling in the regulation of adhesion dynamics of cancer cells.

EXPERIMENTAL PROCEDURES

Antibodies—Anti-human Siglec-9 (goat pAb IgG) was purchased from R&D Systems. Anti-FAK C-terminal antibody

The abbreviations used are: Siglec, sialic acid-binding immunoglobulin-like lectin; AS, astrocytoma; Cas, Crk-associated substrate; CI, cell index; FAK, focal adhesion kinase; ITIM, immunoreceptor tyrosine-based inhibitory motif; RT-CES, real-time cell electronic sensing; GD2, GalNAcβ1,4(NeuAcα2,8 NeuAcα2,3)Galβ1,4Glc-ceramide; GD3, NeuAcα2,8 NeuAcα2,3Galβ1,4Glc-ceramide.
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(rabbit pAb IgG) was purchased from Santa Cruz Biotechnology, and anti-FAK N-terminal antibody (mouse mAb IgG1) was from BD Biosciences. Anti-β-actin (mouse mAb IgG1) was purchased from Sigma-Aldrich. Anti-paxillin (mouse mAb IgG1) was from BD Biosciences. Anti-p130Cas (rabbit IgG, C-20), anti-FAK (rabbit IgG, C-20), anti-phospho-FAK Tyr-576-R (rabbit pAb), FAK Tyr-577-R (rabbit pAb), FAK Tyr-861-R (rabbit pAb), and FAK Tyr-925-R (rabbit pAb) were from Santa Cruz Biotechnology. Anti-phospho-Akt Thr-308 (rabbit mAb IgG), anti-phospho-Akt Ser-473 (rabbit mAb IgG), anti-Akt (rabbit mAb IgG), anti-phospho-p130Cas Tyr-165 (rabbit pAb), and anti-phospho-paxillin (Tyr-118) (rabbit pAb) were from Cell Signaling Technology. Anti-rabbit IgG Alexa Fluor 488 was purchased from Invitrogen. Anti-SHP-1 (rabbit pAb IgG) was purchased from Millipore. See supplemental Experimental Procedures for additional explanation of materials and methods used.

Flow Cytometry—Cell surface expression of Siglec-9Fc-binding ligands was analyzed by FACS CaliberTM (BD Biosciences). Cells were incubated with Siglec-9Fc (5 μg/100 μl in PBS) for 45 min on ice and then stained with FITC-conjugated anti-human IgG secondary antibody (Sigma) for 30 min on ice. Control cells for flow cytometry were prepared using the secondary antibody alone. For quantification of positive cells, the CELLQuest™ program was used.

Neuraminidase Treatment—To analyze whether sialic acids are essential for Siglec-9Fc binding, 1 × 10^6 cells were applied in an FACS tube and incubated with neuraminidase (200 milliunits/ml) from Vibrio cholera (Roche Applied Science) for 1 h at 37 °C. Then Siglec-9 binding was examined by flow cytometry as described below.

Flow Cytometry and Cell Sorting—U937Siglec-9-high and U937mock cells were suspended in cold PBS containing 2% FCS (2 × 10^6 cells/ml). To block non-specific binding, U937 cells were incubated with Fc (5 μg/100 μl PBS) for 15 min in the dark on ice. Cells were then labeled with goat anti-human Siglec-9 antibody (R&D Systems) (10 μg) for 30 min on ice and washed three times with 2% FCS-containing PBS. Ten μg of rabbit anti-goat IgG conjugated with Alexa Fluor 488 was then added and incubated for 30 min. Cells were analyzed using FACS Aria II™ (BD Biosciences). Normal goat IgG was used as a negative control. U937Siglec-9-high cells were sorted from U937 transfected with pcDNA3.1-Siglec-9, and U937Siglec-9-low cells were sorted from U937mock cells. They were used for co-culture experiments as a positive or a negative group.

Productions of Siglec-9-Fc Fusion Proteins—pEE14-Siglec9–3C-Fc plasmid was generated by P. R. Crocker (8), pcDNA 3.1-Fc plasmid was designed in our laboratory. Siglec-9Fc and Fc secreted from HEK293T cells were prepared by DEAE-dextran transfection, and fusion proteins were affinity-purified by protein A-Sepharose (Amersham Biosciences). Protein concentration was measured by the BCA Protein Assay Kit (Thermo).

Cell Lines and Culture—A human astrocytoma cell line AS (9) was maintained in RPMI 1640 medium containing 10% fetal calf serum (FCS) at 37 °C in 5% CO2 incubator. Siglec-9-overexpressing human histiocytic lymphoma (monocyte) U937Siglec-9-high and U937mock cell lines were generated as described (10), both of which were maintained in RPMI 1640 medium containing 10% FCS and G418 (450 μg/ml).

Real-time Cell Electronic Sensing (RT-CES) Test—Cell adhesion and growth were monitored dynamically using the RT-CES system (SP v5.3) (ACEA Bioscience). Cell index (CI) is a parameter used to represent cell adhesion status based on the electrical impedance in gold electrodes at the bottom of plates. CI was collected every 15 min. One × 10^4 AS cells in 100 μl of RPMI 1640 medium containing 10% FCS (regular medium) were seeded into the wells of 16-well e-plates (ACEA Biosience) and cultured for 24 h. Then, U937Siglec-9-high cells (10,000, 25,000, and 50,000) in 100 μl of the regular medium were added. Cells were co-cultured for 52 h or more in a 200-μl volume at 37 °C in 5% CO2 incubator. One × 10^4 of living or fixed U937Siglec-9-high were added in the inhibitor experiments. For the fixation, U937 cells were washed with plain medium and fixed with ethanolacetic acid (95:5) for 20 min at 4 °C. They were washed four times with plain medium and then used for co-culture with AS cells. U937Siglec-9-low cells were used as a negative control. For the stimulation with Siglec-9Fc or Fc proteins, 1 × 10^4 AS cells in 200 μl of the regular medium were seeded in the wells of 16-well e-plates. CI was monitored for 24 h, then 15 μg of Siglec-9Fc or Fc proteins was applied into e-plates, and CI was continuously monitored. All samples are duplicated, and averages of results were used for statistical analysis. To examine whether degradation of FAK was caused by calpain, cells were preincubated with 25 μM MDL-28170 (Calpain Inhibitor III; Bachem AG, Bubendorf, Switzerland).

Co-culture Experiments—AS cells were harvested using 2 mM EDTA/PBS, seeded into 6-well plates at 1 × 10^5/well, and maintained in the regular medium for 24 h at 37 °C. When growing cells covered approximately 70–75% area of the wells, medium was exchanged with plain RPMI 1640 medium for deprivation of FCS. U937Siglec-9-high and U937Siglec-9-low cells were incubated in FCS-free RPMI 1640 medium for 4 h, then 1 × 10^6 of U937Siglec-9-high and U937Siglec-9-low cells were added and cocultured with AS cells. U937 cells were carefully removed without disturbing AS cells by washing twice with plain RPMI 1640 medium. Cells were harvested at 5, 15, and 30 min after co-culture, then lysed with 1 × lysis buffer (Cell Signaling) containing protease inhibitor mixture I (Calbiochem) and 1 mM PMSF (WAKO, Osaka, Japan). Protein concentration was measured by a BCA Protein Assay kit.

Immunoblotting of SHP-1—To examine whether Siglec-9 recruits protein-tyrosine phosphatase 1 (SHP-1) in U937 cells after co-culture with AS cells, U937Siglec-9-high and U937Siglec-9-low co-cultured with AS cells for 15 and 30 min were used as sources of cell lysates. U937 cell lysates (300 μg) were precipitated with 2 μg of human Siglec-9 antibody and protein G-Sepharose (Amershams Biosciences), and the precipitates were served for immunoblotting with anti-SHP-1 antibody.

Wound Healing Assay—Migration activity of AS cells co-cultured with U937Siglec-9-high or U937Siglec-9-low was assessed by scratching assay. AS cells were plated in 6-well plates (1 × 10^5/well) and cultured for 48 h in 10% FCS-containing RPMI 1640 medium. When the cells reached 90% confluency, a scratch was generated using a 200-μl sterile tip. To remove cell debris, each
well was rinsed twice with plain RPMI 1640 medium before co-culture. Then, the cells were overlaid with U937Siglec-9-high or U937Siglec-9-low cells (1 × 10⁶/well) and cultured continuously for 24 h. The wound area images were photographed by a CCD camera (Olympus DP11, Olympus IX70) after carefully removing U937 cells by washing twice with plain RPMI 1640 medium before co-culture.
medium at time 0, 12, and 24 h after the scratching. Wound healing spaces were measured using ImageJ v1.47d software (National Institutes of Health). The wound closure rates were obtained at 3 independent sites in the individual groups.

**Cell Migration Assay with Boyden Chamber**—Matrigel-coated chambers (BD Biosciences) were rehydrated according to the manufacturer’s instruction. AS cells were detached by 2 mM EDTA/PBS and were applied into rehydrated upper chamber at $1 \times 10^6$/ml in RPMI 1640 medium (10% FCS). Three ml of RPMI 1640 medium (10% FCS) was applied into the lower chamber. At 15 min after plating cells, U937<sup>Siglec-9-high</sup> or U937<sup>Siglec-9-low</sup> cells were overlaid at $1 \times 10^6$/ml in RPMI 1640 medium (10% FCS). After co-culture for 24 h at 37 °C, the upper chamber was fixed in 70% ethanol for 5 min, then the upper surface of the chamber was wiped with cotton swabs without disturbing the lower surfaces. The lower surface of the chamber was stained by Giemsa solution, and 10 random fields of the stained membranes were photographed by CCD camera. Then, cell number was counted by a cell counter plugin of ImageJ v1.47d software.

**Immunoblotting**—To examine signaling pathways in AS and other cell lines, cell lysates (15 μg) were separated by SDS-PAGE using 8% gels, and separated proteins were transferred onto a PVDF membrane (Millipore). Blots were blocked with 5% BSA in TBS containing 0.05% Tween 20 (TBST) for more than 6 h at 4 °C. The membrane was first probed with primary antibodies. After being washed, the blots were then incubated with goat anti-rabbit IgGs or horse anti-mouse IgGs conjugated with HRP (1:2000). After washing, bound conjugates were visualized with an ECL™ detection system (PerkinElmer Life Sciences). Band intensities were analyzed with ImageJ and standardized with β-actin.

**RESULTS**

To determine the expression of Siglec-9 ligand-carrier molecules expressed on cancer cells, we performed flow cytometry using 75 human cancer cell lines. Simultaneously, Siglec-9 ligand-carrier molecules were isolated and identified using Siglec-9Fc (8) and mass spectrometry.

The majority of all tested cell lines showed fairly high expression levels of Siglec-9 ligands on the cell surface as shown in supplemental Table S1. According to the score of mean fluorescence intensity, we used a human astrocytoma cell line AS in the following experiments as one of the Siglec-9 ligand-high-expressing cells (Fig. 1A). Proteins identified as Siglec-9 ligand-carrier molecules are summarized in supplemental Table S2. Results of further analysis will be reported elsewhere.

Sialic acid-dependent binding of Siglec-9 was confirmed using neuraminidase as shown in Fig. 1B. To examine the effects of the interaction between immune cells and cancer cells via Siglec-9 on the signaling pathway in both sides of cells, coculture experiments with U937 and AS cells were performed. Stable transfectant cell lines, U937<sup>Siglec-9-high</sup> and U937<sup>Siglec-9-low</sup>, were established as shown in Fig. 1C. These two transfectant lines were apparently similar suspension cells with no attachment to culture dishes. Morphological features of these cells are shown in Fig. 1D. AS cells are adherent cells, and U937 cells are suspension cells (leukemia), and their sizes are quite different, which allowed us to visually distinguish and experimentally separate them from each other very easily.

The co-culture assay was performed by the RT-CES system as described under “Experimental Procedures.” This system could assess the effect of interaction between AS cells and Siglec-9-expressing U937 cells on AS cells by measuring Cl.
Intensities of cell adhesion and spreading can be monitored in a real-time manner. Three kind of stimulants were used, i.e. living U937 cells, fixed ones, and soluble Siglec-9Fc or Fc proteins. The CI of AS cells decreased after co-culture with living U937Siglec-9-high cells at 63 h (5 x 10^5) and 71 h (2.5 x 10^5) of culture. No decrease in CI was found in AS cells co-cultured with U937Siglec-9-low cells (Fig. 2A). When AS cells were co-cultured with fixed U937Siglec-9-high cells, detachment of AS cells was detected immediately after addition of U937 cells. To examine the fates of detached cells, the FCS concentration was increased to 10%, resulting in the reattachment and regrowth of AS cells as shown in RT-CES (Fig. 2B). These features were also observed in a morphological approach (supplemental Fig. S1A). Immediate decrease of cell adhesion was detected when AS cells were treated by soluble Siglec-9Fc (150 μg/ml) in the regular medium, whereas it was gradually restored in CI (Fig. 2C).

Considering the possibility that cell detachment represented cell deterioration due to toxic effects of Siglec-9 binding, immunoblotting for activated Caspase-3 and FACS analysis of Annexin V/propidium iodide was performed (supplemental Fig. S1, B and C). Neither activated Caspase-3 nor apoptosis induction was found in AS cells after co-culture with U937Siglec-9-high cells for 16 h in a FCS-free condition. Higher levels of cell death were found rather in AS cells co-cultured with U937Siglec-9-low (7% versus 1.76%) (supplemental Fig. S1C). Furthermore, SHP-1 was recruited at Siglec-9 in U937Siglec-9-high cells at 15 and 30 min after co-culture with AS cells, whereas no recruitment of SHP-1 was detected in U937Siglec-9-low cells (Fig. 3). This result suggested that Siglec-9 triggered inhibitory signals in U937 via the interaction with its ligands on AS cells as expected. No significant differences in cell growth activity were found during co-culture for 7 days in AS cells (supplemental Fig. S1D).

Cell motility and invasion of AS cells were examined by wound healing assay and cell migration assay, respectively, when they were co-cultured with U937Siglec-9-high or U937Siglec-9-low cells. In the wound healing assay, AS cells co-cultured with U937Siglec-9-high showed significantly higher motility than the negative control and untreated cells (Fig. 4, A and B). In a Boyden chamber assay, AS cells co-cultured with U937Siglec-9-high also showed higher cell invasion than those co-cultured with U937Siglec-9-low (Fig. 4, C and D).

To investigate what occurred in AS cells co-cultured with U937 with high or low levels of Siglec-9, we examined activation of representative proteins. To our surprise, total FAK bands dramatically decreased in AS cells co-cultured with U937Siglec-9-high. Accordingly, all phosphorylation site-specific FAK bands showed definite reduction in the band intensities (Fig. 5, A and B). Minimal changes in FAK and/or phosphorylated FAK were found in AS cells co-cultured with U937Siglec-9-low.
Immunoblotting data of signaling proteins such as Akt, paxillin, and Crk-associated substrate protein (p130Cas) showed that they also underwent reduction in the band intensities (Fig. 5, C–F). In particular, the total Akt band showed marked degradation whereas phosphorylation bands at Thr-308 and Ser-473 did not reduce (Fig. 5, C and D). Bands of p-Akt Thr-308 rather increased in U937Siglec-9-high-treated cells at 15 min.

FIGURE 6. Degradation of FAK and related molecules by addition of Siglec-9Fc. A–D, 5 × 10⁴ AS cells were seeded in 6-well plates and cultured in 10% FCS-containing RPMI 1640 medium for 24 h. Then, the medium was exchanged with FCS-free medium, and cells were incubated for 12 h. Siglec-9Fc or Fc proteins (20 μg) were then added to the wells. After a 15- and 30-min incubation, AS cells were harvested for immunoblotting. Fifteen μg of each protein was applied to 8% SDS-PAGE and then wet transferred onto PVDF membrane, and detected by anti-FAK (A), anti-Akt (B), anti-p130Cas (C) and anti-paxillin (D) antibodies as described in Fig. 5. E, effects of Siglec-9Fc cross-linked by anti-human IgG Fc secondary antibody are shown. To AS cells prepared as in A, preformed Siglec-9Fc and secondary antibody complex (5 or 20 μg each) was added. Anti-human IgG F(ab')₂ fragment (Sigma) was used by mixing in 200 μl of PBS before incubation for 2 h at room temperature. Fc fusion protein instead of Siglec-9Fc was used as a negative control. After a 15- and 30-min incubation, AS cells were washed and harvested for immunoblotting. Fifteen μg of each protein was applied to 8% SDS-PAGE, then detected by anti-FAK (N-terminal) antibody. *, nonspecific band; deg, degradation products.

Immunoblotting data of signaling proteins such as Akt, paxillin, and Crk-associated substrate protein (p130Cas) showed that they also underwent reduction in the band intensities (Fig. 5, C–F). In particular, the total Akt band showed marked degradation whereas phosphorylation bands at Thr-308 and Ser-473 did not reduce (Fig. 5, C and D). Bands of p-Akt Thr-308 rather increased in U937Siglec-9-high-treated cells at 15 min.

FAK after normalization with band intensities of β-actin. C, results of immunoblotting for total Akt and phosphorylated forms at Thr-308 and Ser-473 in AS cells treated as in A. D, results of the densitometric analysis of total Akt and phosphorylated Akts. Results are shown as described in B. E, results of immunoblotting for p130Cas and paxillin in AS cells treated as in A and C. F, results of the densitometric analysis of total p130Cas and paxillin and phosphorylated forms as shown in B and D. These experiments were repeated at least three times with similar results.
Phosphorylated bands of paxillin and p130Cas also showed no changes (Fig. 5, E and F). No significant differences were found during co-culture with U937<sup>Siglec-9-high</sup>. By the treatment of AS cells with soluble Siglec-9Fc, similar degradation of FAK, Akt, p130Cas, and paxillin was also found, whereas Fc protein alone showed no effects (Fig. 6, A–D). When a complex of Siglec-9Fc with anti-human IgG F(ab′)<sub>2</sub> was used, stronger degradation of FAK was found than in A (Fig. 6E). No significant differences in band intensities of total ERKs and Src and their phosphorylation forms were found between cells co-cultured with U937<sup>Siglec-9-high</sup> and those with U937<sup>Siglec-9-low</sup> (supplemental Fig. S2, A and B).

To clarify spatiotemporal dynamics of the protein degradation and its implication in cell behaviors, time courses of the degradation and intracellular localization of FAK and p-Akt were analyzed by immunoblotting and immunocytochemistry, respectively. As shown in Fig. 7, FAK levels were restored at 12–24 h after the addition of FCS, whereas p-Akt Thr-308 levels increased in an earlier stage (~60 min), probably reflecting increased cell motility in AS cells treated with U937<sup>Siglec-9-high</sup>.

Immunocytochemistry revealed that FAK staining was markedly reduced in U937<sup>Siglec-9-high</sup>-treated AS cells, and remaining FAK was found only one side of cytoplasm compared...
with AS cells treated with U937\textsuperscript{Siglec-9-low}, in which FAK was distributed highly and broadly in the cytoplasm (Fig. 8A). Furthermore, simultaneous staining of FAK and p-Akt Thr-308 revealed that these two molecules were localized at the same side of cells, probably the front region of cells (Fig. 8B). Details are shown in supplemental Fig. S4.

Degradation of FAK through Siglec-9 was examined in another cell lines, i.e. an ovarian cancer cell line (HEY) and a
glioma cell line (U251). They showed similar FAK degradation during co-culture with U937\textsuperscript{Siglec-9-high} (Fig. 9A) which indicated universality of the results with AS cells. Results of RT-CES also showed similar reduction of CI and subsequent restoration after FCS treatment as observed in AS cells (Fig. 9B). Sialic acid dependence of these protein degradations was examined by pretreatment of AS cells with neuraminidase before co-culture, resulting in the reduced degradation as expected (supplemental Fig. S3).

To clarify whether the dynamic changes of bands in FAK, Akt, and so on during the co-culture of AS cells with U937\textsuperscript{Siglec-9-high} cells were protein degradation due to proteolytic enzymes, blocking of calpain was performed because calpain seemed to be the most likely protease responsible for the cleavage of FAK (11). In RT-CES, AS cells treated with U937\textsuperscript{Siglec-9-high} and a calpain inhibitor MDL-28170 caused apparently less reduction of CI than those with U937\textsuperscript{Siglec-9-high} alone (Fig. 10A). This was particularly clear when AS cells were treated with fixed U937\textsuperscript{Siglec-9-high} and MDL-28170. These cells showed almost same pattern (no reduction of CI) asuntreated cells (Fig. 10B), whereas AS cells treated with fixed U937\textsuperscript{Siglec-9-high} alone showed definite CI reduction. This was also the case when AS cells were treated by Siglec-9-Fc (Fig. 10C).

By the treatment of AS cells with 25 \(\mu\)M MDL-28170 before co-culture with U937\textsuperscript{Siglec-9-high}, degradation of FAK was completely suppressed (Fig. 11, A and B). This was also the case for Akt, p130Cas, and paxillin (Fig. 11, C and D), strongly suggesting that calpain was responsible for the degradation of these molecules. All of these results are summarized in Fig. 12.

**DISCUSSION**

The majority of past studies on Siglec-mediated signaling have been done with ITIM-associated inhibitory signals in immune cells. There have been no reports on the signaling in cancer cells that express counterreceptors with sugar chains recognized by Sigslec. In this study, we demonstrated, for the first time, that Siglec-9 triggered a signal, leading to degradation of FAK, Akt, and related molecules in cancer cells. The most novel point in this study is that cancer cells receive not death signals, but a sort of activation signal via interaction between Siglec-9 on immune cells and its counterreceptors on cancer cells. Furthermore, it was clearly shown that all observed differences in Figs. 2–5 were due to the interaction between Siglec-9 and sialylglycoconjugates/carrier proteins because the presence/absence of Siglec-9 on U937 was the sole difference in our system, and the removal of sialic acids clearly canceled Siglec-9 binding and FAK degradation.

FAK is a tyrosine kinase playing multiple functions in the cell signaling for cell adhesion (12), proliferation (13), migration (14), and cancer metastasis (12, 13). FAK is present mainly at the focal adhesion and plays essential roles by interacting with various signaling molecules such as p130Cas, paxillin, and Src family kinases (15). We have investigated the roles of FAK in the enhanced malignant properties such as cell proliferation (16) and cell adhesion to extracellular matrix (17) in malignant melanomas expressing ganglioside GD3. Apoptosis induction of small cell lung cancer cells by anti-ganglioside GD2 monoclonal antibodies via dephosphorylation of FAK was also reported by us (7). In addition to enhanced adhesion to extracellular matrix, increased migration and cell motility are also a hallmark of cancer cells (18). FAK has been considered to regulate cell migration by orchestrating signals between growth factor receptors and integrins (19). Although FAK is important in the cell adhesion in association with integrins, it is also essential for the regulation of focal adhesion disassembly (20). Degradation of FAK might be important for the promotion of cell migration by regulation of adhesion dynamics (11).

In our results, FAK underwent rapid degradation in cancer cells after co-culture with Siglec-9-expressing cells. Represent-
ative tyrosine-phosphorylated forms of FAK also degraded along with total FAK. However, cell migration and invasion activities increased after co-culture. These paradoxical results might indicate the differences in the roles of FAK depending on the spatial and temporal dynamics of cell adhesion and migration as shown in Fig. 12. Signaling molecules other than FAK showed sustained levels of individual phosphorylated forms during co-culture, suggesting partially overlapping activation of those molecules with FAK degradation. Among molecules that showed sustained activated levels, we focused on p-Akt Thr-308 because of its increased phosphorylation in the early phase of co-culture, and we could show its localization in the front side of AS cells as well as remaining FAK. These results should explain, at least partly, mechanisms for the increased migration of AS cells undergoing FAK degradation as shown in Fig. 12. Increased activation of Akt Thr-308 as shown in Fig. 5, C and D, might also be involved in the resistance to apoptosis in AS cells (21). Details in the spatiotemporal regulation of FAK molecules as well as its related molecules in the cell migration remain to be investigated.

Results obtained in this study suggested that proteolytic enzymes should be activated and exert for the transient degra-
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