Binding of the Sialic Acid-binding Lectin, Siglec-9, to the Membrane Mucin, MUC1, Induces Recruitment of β-Catenin and Subsequent Cell Growth

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Background: MUC1 plays a role in mediation of signaling initiated by growth factors.
Results: Siglec-9-positive cells are associated with MUC1-positive tumor cells in tumor tissues, and Siglec-9 binds to MUC1.
Conclusion: MUC1-mediated signaling occurs by direct binding of Siglec-9 to MUC1.
Significance: Multiple signaling pathway through MUC1 can be advantageous to adjust to various conditions of tumor microenvironments.

Because MUC1 carries a variety of sialoglycans that are possibly recognized by the siglec family, we examined MUC1-binding siglec and found that Siglec-9 prominently bound to MUC1. An immunochemical study showed that Siglec-9-positive immune cells were associated with MUC1-positive cells in human colon, pancreas, and breast tumor tissues. We investigated whether or not this interaction has any functional implications for MUC1-expressing cells. When mouse 3T3 fibroblast cells and a human colon cancer cell line, HCT116, stably transfected with MUC1cDNA were ligated with recombinant soluble Siglec-9, β-catenin was recruited to the MUC1 C-terminal domain, which was enhanced on stimulation with soluble Siglec-9 in dose- and time-dependent manners. A co-culture model of MUC1-expressing cells and Siglec-9-expressing cells mimicking the interaction between MUC1-expressing malignant cells, and Siglec-9-expressing immune cells in a tumor microenvironment was designed. Brief co-incubation of Siglec-9-expressing HEK293 cells, but not mock HEK293 cells, with MUC1-expressing cells similarly enhanced the recruitment of β-catenin to the MUC1 C-terminal domain. In addition, treatment of MUC1-expressing cells with neuraminidase almost completely abolished the effect of Siglec-9 on MUC1-mediated signaling. The recruited β-catenin was thereafter transported to the nucleus, leading to cell growth. These findings suggest that Siglec-9 expressed on immune cells may play a role as a potential counterreceptor for MUC1 and that this signaling may be another MUC1-mediated pathway and function in parallel with a growth factor-dependent pathway.

Membrane-bound mucins have been considered to play important biological roles in cell-cell and cell-matrix interactions (1, 2). Aberrant expression of MUC1 on various cancer cells is associated with a poor prognosis. The MUC1 protein is translated as a single polypeptide, which is cleaved in the endoplasmic reticulum, yielding N- and C-terminal subunits that form a heterodimeric complex bound through noncovalent interactions (3, 4). The N-terminal ectodomain (MUC1-ND) 2 consists of variable numbers of 20-amino acid tandem repeats that are extensively O-glycosylated (5). The C-terminal domain (MUC1-CD) anchors the MUC1-ND to the cell surface.

It has been reported that MUC1 associates with the EGF family of growth factor receptor kinases in some human carcinoma cells and mouse mammary gland and potentiates EGF-dependent signal transduction (6–9). In addition to the EGF family, MUC1 also interacts with FGF receptor-3 (FGFR-3) (10). Treatment of breast cancer cells with FGF increases the binding of MUC1-CD to β-catenin and targeting of MUC1-CD and β-catenin to the nucleus. Dysregulation of β-catenin is of great importance as to the development of diverse tumor malignancies (11). However, studies on signaling through MUC1 following direct ligation of MUC1-ND with an endogenous ligand have been limited except in the case of ICAM-1 (12, 13).

MUC1-ND has an extended structure because it carries a large number of sialylated O-glycans. This extended structure is expected to act as a particularly effective scaffold for the presentation of oligosaccharide chains to lectins, thereby promoting interactions between cancer cells and appropriate lectin-expressing cells. It has been reported that MUC1 is a natural ligand of endogenous Siglec-1 (14) and galectin-3 (15, 16) and that the binding of galectin-3 increases the adhesion between cancer cells and endothelial cells and the aggregation of tumor cells. However, these studies provided no insights to the downstream signaling by MUC1 after binding of these lectins. In many tumor tissues, infiltrating immune cells such as macrophages are associated with MUC1-positive cells in human colon, pancreas, and breast tumor tissues. We investigated whether or not this interaction has any functional implications for MUC1-expressing cells.

2 The abbreviations used are: ND, N-terminal domain; CD, C-terminal domain; EGF, EGF receptor; FGFR-3, FGF receptor-3; MAM, Maackia amurensis mitogen; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; ICAM-1, intercellular adhesion molecule-1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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phages are commonly found closely associated with tumor cells (17), but little is known concerning the nature or significance of the adhesion and/or recognition molecules involved in such cellular interaction. Immune cells express various siglec including Siglec-1. The Siglec family are sialic acid-binding, immunoglobulin-like lectin, most of which are expressed on immune cells (18). Recently, we demonstrated that mucins bound to Siglec-2 (19) and -9 (20), which are expressed on B cells and dendritic cells, respectively, leading to negative immunomodulation in these cells. These facts raised the possibility that this direct binding of siglecs to MUC1 may bring about signal transduction through MUC1. To assess this signal transduction, we used mouse 3T3 fibroblast cells transfected with MUC1cDNA in addition to epithelial cancer cell lines, because 3T3/MUC1 cells may be useful to clarify the function of MUC1 in cells lacking tumor-associated genetic and epigenetic changes. In the present study, we demonstrated that direct binding of Siglec-9 to MUC1-ND triggers recruitment of β-catenin to MUC1-CD, followed by nuclear transport in the manner like growth factor-triggered MUC1-CD mediated signaling. Thus, this MUC1-mediated signaling may work cooperatively with a growth factor-triggered pathway.

**MATERIALS AND METHODS**

**Cells**—Mouse fibroblast NIH3T3 cells, a human colon cancer cell line, HCT116, a human embryonic kidney cell line, HEK293, and a human alveolar cell line, A549, were obtained from the American Type Culture Collection. 3T3/MUC1 and HCT116/MUC1 cells were prepared by introducing MUC1cDNA into mouse 3T3 fibroblasts and human colon cancer HCT116 cells, respectively, as described previously (9), and 3T3 and HCT116 mock cells were prepared by transfection of the empty vector. 3T3/mock and 3T3/MUC1 cells were cultured in RPMI 1640 medium containing 5 mM glucose and 10% fetal calf serum. HCT116/mock and HCT116/MUC1 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. HEK293 cells expressing Siglec-9 (HEK293/Siglec-9) and mock cells (HEK293/mock) were obtained by transfection of Siglec-9 cDNA and the empty vector, respectively, and cultured in Dulbecco’s modified Eagle’s medium containing 5 mM glucose and 10% fetal calf serum. A549 cells were cultured in F-12 K medium containing 10% fetal calf serum, 1% penicillin, and 1% streptomycin.

**Immunostaining**—3T3/MUC1 cells (5 × 10^3 cells) were cultured in the presence or absence of recombinant soluble Siglec-9 (sSiglec-9) (R&D, 2.5 µg/ml) at 37 °C for 1 h. After fixation with PBS containing 4% paraformaldehyde, cells were treated with PBS containing 0.1% Triton-X-100 and 10% goat serum, and then with mouse anti-β-catenin antibodies (Santa Cruz Biotechnology) or a control IgG at room temperature for 2 h. After washing with PBS, the cells were stained with Alexa Fluor 488-labeled goat anti-mouse IgG antibodies (Molecular Probes) and propidium iodide. Slides were mounted using ProLong Gold Antifade reagents (Invitrogen). Distribution of MUC1-CD and β-catenin in the nucleus was examined using the same procedure as described above with combinations of mouse anti-β-catenin antibodies and Alexa Fluor 488-labeled goat anti-mouse IgG antibodies, and Armenian hamster anti-MUC1 Ab5 antibodies (Neo Marker) and DyLight 594-labeled goat anti-hamster IgG antibodies (BioLegend), respectively.

Sections of paraffin-embedded tumor and nonmalignant tissues were deparaffinized through xylene and alcohol gradients. Antigen retrieval was performed by treatment with 0.01 M citric acid buffer at 100 °C for 15 min. After washing with PBS, sections were blocked with 5% BSA-PBS and then with mouse anti-MUC1-ND antibodies (BD Biosciences) and rabbit anti-Siglec-9 antibodies (ABGENT) or a control IgG at 4 °C for 24 h. After washing with PBS, the sections were stained with Alexa Fluor 488-labeled goat anti-mouse IgG antibodies, Alexa Fluor 594-labeled goat anti-rabbit IgG antibodies (Molecular Probes), and DAPI. Specimens of tumor and adjacent nonmalignant tissues were obtained from cancer patients in accordance with the protocol approved by Osaka City University.

**Flow Cytometry**—3T3 and HCT116 mock cells, and their MUC1 transfectants (1 × 10^6 cells) were treated with mouse anti-MUC1-ND antibodies or a control mouse IgG in 0.5% BSA-PBS at 4 °C for 1 h. After washing with 0.5% BSA-PBS, the cells were incubated with FITC-labeled goat anti-mouse IgG antibodies (Zymed Laboratories Inc.). 3T3/MUC1 cells (5 × 10^5 cells) were incubated with rabbit anti-EGFR antibodies (Santa Cruz Biotechnology), rabbit anti-FGFR-3 antibodies (Santa Cruz Biotechnology), or a control IgG at 4 °C for 2 h. After washing as described above, the cells were incubated with Alexa Fluor 488-labeled goat anti-rabbit IgG antibodies (Molecular Probes). HEK293/Siglec-9 and HEK293/mock cells (1 × 10^6 cells) were incubated with mouse anti-Siglec-9 antibodies (R&D Systems) or a control mouse IgG in 0.5% BSA-PBS at 4 °C for 1 h. After washing with 0.5% BSA-PBS, the cells were incubated with FITC-labeled rabbit anti-mouse IgG antibodies (Zymed Laboratories Inc.). 3T3/MUC1 and HCT116/MUC1 cells (1 × 10^6 cells) were also treated with biotin-labeled MAM (Vector Laboratories) at 4 °C for 2 h and then with FITC-labeled streptavidin (BD Biosciences). All cells were analyzed with a FACS Calibur (BD Biosciences).

**Binding of Recombinant Siglecs to MUC1**—Fc-tagged Siglec-3, -5, -9, and His-tagged Siglec-1 (R&D Systems, 2 µg of protein) were added to lysates (1 mg of protein) of 3T3/MUC1 cells, followed by mixing at 4 °C for 2 h. Proteins bound to siglecs were pulled down with protein G-Sepharose 4 Fast Flow (GE Healthcare) or nickel-nitritolriacetic acid-agarose (Qiagen). MUC1 was purified from the conditioned medium of 3T3/MUC1 cells by gel filtration and CsCl density gradient ultracentrifugation as described previously (21).

Universal Bind 96-well plates (Corning) were coated with MUC1 (0.5 µg of protein) in PBS or PBS (control) at 4 °C for 2 h, followed by blocking with 2.5% BSA-PBS. Fc-tagged Siglec-9 (1 µg of protein) was added to the plates, followed by incubation for 1 h, and then washing with 50 mM sodium phosphate buffer, pH 7.5, 0.2 M NaCl, and 0.05% Tween 20. Bound Fc-tagged Siglec-9 was estimated with HRP-conjugated protein G (GE Healthcare) and a TMB peroxidase substrate system (Nacalai Tesque).
Siglec-9-triggered Recruitment of β-Catenin to MUC1

Treatment of MUC1-expressing Cells with sSiglec-9 and Siglec-9-expressing Cells—3T3/MUC1 and 3T3/mock cells (1 × 10^6 cells) were cultured in serum-free RPMI 1640 medium for 1 h prior to analysis, and then after washing with the same medium, the cells were treated with sSiglec-9 (1–5 μg of protein) for the indicated times in the same medium. When the phosphorylation of β-catenin was examined, these cells were treated with sSiglec-9 (5 μg of protein) for 40 min. When MUC1-mediated signaling was analyzed after co-incubation with Siglec-9-expressing cells, the following procedures were performed. 3T3/MUC1 cells and HCT116/MUC1 cells were cultured in serum-free RPMI 1640 medium and DMEM, respectively, for 1 h prior to analysis. HEK293/Siglec-9 and HEK293/mock cells were also cultured in serum-free DMEM for 1 h prior to analysis. After washing all the cells with serum-free RPMI 1640 medium, suspensions of 3T3/MUC1 or HCT116/MUC1 cells (1 × 10^6 cells) were mixed with suspensions of HEK293/Siglec-9 or HEK293/mock cells (1 × 10^6 cells) in serum-free RPMI 1640 medium and then rotated gently at 37 °C for 20 min.

Immunoprecipitation—3T3 and HCT116 mock cells, their MUC1 transfectants, and A549 cells were solubilized with a solubilizing solution (1% Nonidet P-40, 10 mM Tris–HCl, pH 8.0, 0.14 M NaCl, 1 mM EDTA, 1 mM PMSF, and protease inhibitor mixture (Nacalai Tesque). MUC1 was immunoprecipitated from lysates (500 μg of protein) of 3T3/MUC1, HCT116/MUC1, and A549 cells using mouse anti-MUC1-ND antibodies and protein G-Sepharose 4 Fast Flow. EGFR and FGFR-3 were immunoprecipitated from the cell lysates of 3T3/MUC1 and HCT116/MUC1 cells with rabbit anti-EGFR antibodies (Cell Signaling) and anti-FGFR-3 antibodies, respectively, and protein G-Sepharose 4 Fast Flow. To examine the co-immunoprecipitation of MUC1-CD with β-catenin, MUC1-CD or β-catenin was immunoprecipitated using Armenian hamster anti-MUC1 Ab5 antibodies or mouse anti-β-catenin antibodies and protein G-Sepharose 4 Fast Flow.

Western Blot Analyses—Lysates of 3T3 and HCT116 mock cells, their transfectants, and neuraminidase-treated transfectants, immunoprecipitates, and proteins pulled down with siglecs as described above were subjected to SDS-PAGE followed by Western blotting using Immuno-Blot PVDF membranes (Bio-Rad), except for the detection of MUC1-ND. In the case of MUC1-ND, Zeta-Probe Blotting membranes (Bio-Rad) were used. Detection of MUC1-ND, β-catenin, phosphorylated β-catenin, histone 2B, and IßB-α was performed using mouse anti-MUC1-ND, anti-β-catenin, anti-phosphorylated β-catenin (Santa Cruz Biotechnology), anti-histone 2B (Cell Signaling), and anti-IßB-α (Santa Cruz Biotechnology) antibodies, respectively, and HRP-labeled rabbit anti-mouse IgG antibodies (Zymed Laboratories Inc.). FGFR-3, EGFR, c-Src, Lyn, Lck, and c-myc were detected using rabbit anti-FGFR-3, anti-EGFR (Cell Signaling), anti-c-Src (Cell Signaling), anti-Lyn (Cell Signaling), anti-Lck (Santa Cruz Biotechnology), and anti-c-myc (Cell Signaling) antibodies, and HRP-labeled goat anti-rabbit IgG antibodies (Zymed Laboratories Inc.). MUC1-CD was detected using Armenian hamster anti-MUC1 Ab5 antibodies and HRP-labeled goat anti-hamster IgG antibodies (Santa Cruz Biotechnology). The intensity of bands was determined using ImageJ software (National Institutes of Health).

Cell Fractionation—Cell fractionation was performed basically according to Halwani et al. (22).

Neuraminidase Treatment—3T3/MUC1 and HCT116/MUC1 cells (1 × 10^6 cells) were treated with 50 milliunits of neuraminidase (Arthrobacter ureafaciens, Nacalai Tesque) in serum-free RPMI 1640 medium at 37 °C for 1 h.

Cell Growth—3T3/MUC1 cells (2 × 10^3 cells) were treated with or without sSiglec-9 for 20 min and then cultured in RPMI 1640 medium supplemented with 2 mM l-glutamine and 10% fetal calf serum until 72 h. Cell growth was determined by means of the MTT assay. 3T3/MUC1 cells (1 × 10^6 cells) treated with or without sSiglec-9 and cultured as described above were solubilized at 24 h after treatment with sSiglec-9, and then subjected to SDS-PAGE, followed by Western blotting and detection with rabbit anti-c-myc antibodies.

RESULTS

Siglec-9 Binds to MUC1—MUC1cDNA was introduced into mouse 3T3 fibroblasts, and stable transfectants were obtained. MUC1 expression was confirmed by flow cytometry (Fig. 1A).
There was no detectable MUC1 expression on cells transfected with the empty vector. A cell lysate was subjected to SDS-PAGE, followed by Western blotting, and detection with anti-MUC1-ND and -CD antibodies. The bands of MUC1-ND and -CD were detected at the positions of ~250 and 15–25 kDa, respectively. Multiple bands of MUC1-CD are considered to be due to different levels of N-glycosylation, as described previously (23) (data not shown).

To examine the binding of siglec9 to MUC1, soluble tagged Siglec-1, -3, -5, and -9 were added to lysates of 3T3/MUC1 cells, followed by mixing. Proteins associated with these siglec9 were pulled down and then subjected to SDS-PAGE, followed by Western blotting. MUC1-ND was detected in all precipitates pulled down with siglec9 (Fig. 1B). Because MUC1 was pulled down predominantly with Siglec-9 (Fig. 1B, lane b), further experiments were performed using recombinant soluble Siglec-9 (sSiglec-9). Furthermore, MUC1-ND was prepared from culture medium of 3T3/MUC1 cells and immobilized on a microplate. Binding of sSiglec9 to MUC1-ND was also confirmed by means of a plate assay (Fig. 1C).

Infiltrating Siglec-9-positive Cells Are Observed in MUC1-positive Tumor Tissues—To determine whether or not Siglec-9-expressing cells are actually associated with MUC1-positive cells in human tumor tissues, colon, breast, and pancreas tumor tissues were immunostained with anti-Siglec-9 and anti-MUC1-ND antibodies as described under “Materials and Methods” (Fig. 2). Siglec-9-positive cells were frequently observed in MUC1-positive colon, breast, and pancreatic tissues, whereas a lower level of MUC1 was expressed and a smaller number of Siglec-9-positive cells were observed in nonmalignant colon tissues compared with tumor tissues. A similar staining pattern was obtained for other nonmalignant tissues (data not shown). In tumor tissues, many Siglec-9-positive cells were closely associated with MUC1-positive cells, suggesting an interaction between Siglec-9 and MUC1.

FGFR-3 Is Not Associated Physically with MUC1 in 3T3/MUC1 Cells—It has been reported that EGF and FGF receptors are associated with MUC1 in some human carcinoma cells and mouse mammary glands and that ligation with each growth factor induces the binding of β-catenin to MUC1 (7, 9, 10). Considering these reports, we examined the expression of these receptors on 3T3/MUC1 cells by flow cytometry. As shown in Fig. 3A, 3T3/MUC1 cells expressed FGFR-3 but not EGFR. To determine whether or not MUC1 associates with FGFR-3, a co-immunoprecipitation experiment was performed. From a lysate of 3T3/MUC1 cells, MUC1-ND was immunoprecipitated and then subjected to SDS-PAGE followed by Western blotting. FGFR-3 was not detected in the immunoprecipitate obtained with anti-MUC1-ND antibodies (Fig. 3B, lane b). In a reciprocal experiment, MUC1-ND was not co-immunoprecipitated with anti-FGFR-3 antibodies (Fig. 3B, lane c). Because ligation of Siglec-9 or FGF may have some effect on the association between MUC1 and FGFR-3, cells were treated in the presence or absence of FGF or sSiglec-9, and then cell lysates were prepared. The immunoprecipitates derived from a cell lysate with either anti-FGFR-3 antibodies or anti-MUC1-ND antibodies were subjected to SDS-PAGE followed by immunoblotting with antibodies to the other. Co-immunoprecipitation of MUC1 and FGFR-3 was not observed, irrespective of ligation with FGF or sSiglec-9, as shown in Fig. 3, C and D. Thus, it appears likely that MUC1 does not function in signaling from FGF receptors in 3T3/MUC1 cells. Furthermore, a similar experiment was performed using HCT116/MUC1 cells. MUC1 or EGFR was immunoprecipitated from a lysate of HCT116/MUC1 cells and then analyzed as described above. Although MUC1 and EGFR were detected in the immunoprecipitates with anti-MUC1-ND and anti-EGFR antibodies, respectively (Fig. 3E, lanes a and d), EGFR and MUC1 were not co-immunoprecipitated with MUC1 and EGFR, respectively (Fig. 3E, lanes b and c).

MUC1-mediated Signaling Is Initiated Directly by Binding of sSiglec-9 to MUC1—It has been reported that MUC1-mediated signaling originates indirectly through ligation of growth factors such as EGF and FGF with the receptors that are associated with MUC1 on the cell surface (6–11). We were interested in determining whether or not direct binding of sSiglec-9 to MUC1 induces the recruitment of β-catenin to MUC1-CD. 3T3/mock and 3T3/MUC1 cells were treated with sSiglec-9 for 20 min. Then, MUC1-CD was immunoprecipitated from cell lysates and subjected to SDS-PAGE followed by Western blotting. β-Catenin was co-immunoprecipitated with MUC1-CD when 3T3/MUC1 cells, but not 3T3/mock cells, were treated with sSiglec-9 (Fig. 4A, lanes a–d). In a reciprocal experiment, MUC1-CD was detected in the immunoprecipitate with anti-β-catenin antibodies only when 3T3/MUC1 cells were treated with sSiglec-9 (Fig. 4A, lanes e and f). Similar experiments were performed using a human colon cancer cell line, HCT116, transfected with MUC1cDNA, and a human alveolar epithelial cell line, A549. β-Catenin was similarly recruited to MUC1-CD through ligation with sSiglec-9 (Fig. 4A, lanes g–j). This suggests that recruitment of β-catenin was triggered by the binding of Siglec-9 to MUC1.

Next, we examined the time- and dose-dependence of recruitment of β-catenin to MUC1-CD in 3T3/MUC1 cells. After treatment with sSiglec-9 for various times or treatment with various amounts of sSiglec-9 for 20 min, MUC1-CD was immunoprecipitated from cell lysates, followed by SDS-PAGE and immunoblotting (Fig. 4, B and C). The intensity of the bands was determined as described under “Materials and Methods.” The level of recruited β-catenin peaked at 20 min and increased in a dose-dependent manner (Fig. 4, D and E). It has been reported that c-Src, Lyn, and Lck can bind and phosphorylate MUC1-CD, facilitating the recruitment of β-catenin (24–26). First, we examined the expression of c-Src, Lyn, and Lck in 3T3/MUC1 cells. A lysate of 3T3/MUC1 cells was subjected to SDS-PAGE, followed by Western blotting and detection with each antibody. As shown in Fig. 4F, c-Src and Lck but not Lyn were detected in 3T3/MUC1 cells (lane a). Furthermore, 3T3/MUC1 cells were treated with or without sSiglec-9 for 20 min, and a lysate was prepared. MUC1-CD was immunoprecipitated and subjected to SDS-PAGE, followed by Western blotting. Co-immunoprecipitated kinase was revealed to be c-Src, and the level of co-immunoprecipitated c-Src was elevated through ligation with sSiglec-9 (Fig. 4F, lanes b and c), maybe leading to enhance β-catenin recruitment to MUC1-CD as described above. To further confirm the involvement of
c-Src, 3T3/MUC1 cells were stimulated with sSiglec-9 in the presence or absence of Src family kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2). The level of β-catenin recruited to MUC1-CD decreased by approximately 60% on treatment with PP2, indicating that recruitment of β-catenin to MUC1-CD requires a c-Src in 3T3/MUC1 cells (Fig. 4, G and H).

**MUC1-mediated Signaling Is Also Initiated by Membrane-bound Siglec-9**—To see whether signaling takes place through cellular interaction between MUC1- and Siglec-9-expressing...
cells, Siglec-9-expressing cells were established. Siglec-9 cDNA was introduced into HEK293 cells, and the expression of Siglec-9 was confirmed with a flow cytometer (Fig. 5A). Furthermore, to confirm that MUC1-mediated signaling is sialic acid-dependent, 3T3/MUC1 and HCT116/MUC1 cells were treated with neuraminidase as described under "Materials and Methods." Removal of sialic acid residues from the cell surface was demonstrated by the decrease of Maackia amurensis mitogen (MAM) binding (Fig. 5B). Furthermore, lysates of 3T3/MUC1 and HCT116/MUC1 cells treated with or without neuraminidase were subjected to SDS-PAGE, followed by Western blotting and detection with anti-MUC1-ND antibodies (lanes a and c) and anti-FGFR-3 antibodies (lanes b and d). C and D, after treatment of 3T3/MUC1 cells with FGF (C) or sSiglec-9 (D), the same procedure was performed as described in B. Each lane contained a sample treated similarly to that in B. E, MUC1 (lanes a and b) and EGFR (lanes c and d) were immunoprecipitated with mouse anti-MUC1-ND antibodies and rabbit anti-EGFR antibodies, respectively, from a lysate of HCT116/MUC1 cells, and then subjected to SDS-PAGE. After Western blotting, MUC1-ND (lanes a and c) and EGFR (lanes b and d) were detected with the same antibodies as described above.

FIGURE 3. Analyses of proteins co-immunoprecipitated with MUC1. A, expression of FGFR-3 and EGFR was analyzed by flow cytometry as described under "Materials and Methods." Broken line, control IgG; solid line, rabbit anti-FGFR-3 antibodies or rabbit anti-EGFR antibodies. B, MUC1 (lanes a and b) and FGFR-3 (lanes c and d) were immunoprecipitated (IP) with mouse anti-MUC1-ND antibodies and rabbit anti-FGFR-3 antibodies, respectively, from a lysate of 3T3/MUC1 cells, and the immunoprecipitate was subjected to SDS-PAGE, followed by Western blotting and detection with anti-MUC1-ND antibodies (lanes a and c) and anti-FGFR-3 antibodies (lanes b and d). C and D, after treatment of 3T3/MUC1 cells with FGF (C) or sSiglec-9 (D), the same procedure was performed as described in B. Each lane contained a sample treated similarly to that in B. E, MUC1 (lanes a and b) and EGFR (lanes c and d) were immunoprecipitated with mouse anti-MUC1-ND antibodies and rabbit anti-EGFR antibodies, respectively, from a lysate of HCT116/MUC1 cells, and then subjected to SDS-PAGE. After Western blotting, MUC1-ND (lanes a and c) and EGFR (lanes b and d) were detected with the same antibodies as described above.

Phosphorylation of β-Catenin Is Down-modulated with Ligation of sSiglec-9 with MUC1, and β-Catenin Is Transported to the Nucleus—3T3/MUC1 and 3T3/mock cells were treated with or without sSiglec-9 for 40 min, and then the cell lysates were subjected to SDS-PAGE, followed by Western blotting and detection with anti-phosphorylated β-catenin antibodies. Phosphorylated β-catenin was significantly reduced by the treatment with sSiglec-9 in 3T3/MUC1 cells, but it was not
Siglec-9-triggered Recruitment of β-Catenin to MUC1

**FIGURE 4. Recruitment of β-catenin to MUC1 on stimulation with sSiglec-9.** A, 3T3/mock (lanes a and b), 3T3/MUC1 (lanes c–f), HCT116/MUC1 (lanes g and h), and A549 (lanes i and j) cells (1 × 10^6 cells) were stimulated with or without sSiglec-9 for 20 min, and then MUC1-CD (lanes a–d, g–j) and β-catenin (lanes e and f) were immunoprecipitated (IP) with Armenian hamster anti-MUC1 Ab5 antibodies and mouse anti-β-catenin antibodies, respectively, from the cell lysates. MUC1-CD and β-catenin were detected with the same antibodies as described above after SDS-PAGE and Western blotting (IB). B, MUC1-CD was immunoprecipitated as described above from a lysate of 3T3/MUC1 cells (1 × 10^6 cells) stimulated with sSiglec-9 for 0–40 min. After SDS-PAGE and Western blotting, co-immunoprecipitated β-catenin was detected. C, 3T3/MUC1 cells (1 × 10^6 cells) were stimulated with sSiglec-9 (0–5 μg of protein/ml) for 20 min, and the same procedure was performed as described in B. D, the intensity of the band of β-catenin in B was estimated with ImageJ software. The level of β-catenin relative to that of MUC1-CD was compared. The value obtained in the experiment in which 3T3/MUC1 cells were treated with sSiglec-9 for 0 min was taken as 1 (mean ± S.D., error bars, n = 5; *p < 0.05). E, the level of β-catenin in C was estimated as described in D, and the intensity of β-catenin relative to that of MUC1-CD was compared. The value obtained in the experiment in which 3T3/MUC1 cells were treated with sSiglec-9 in the absence of PP2 (10 μM) for 20 min was taken as 1 (mean ± S.D., n = 3; *p < 0.01).

The value obtained in the experiment in which 3T3/MUC1 cells were treated without sSiglec-9 for 20 min was taken as 1. F, a lysate of 3T3/MUC1 cells (lane a) and immunoprecipitates with Armenian hamster anti-MUC1 Ab5 antibodies from lysates of 3T3/MUC1 cells (1 × 10^6 cells) treated with (lane c) or without (lane b) sSiglec-9 for 20 min were subjected to SDS-PAGE, followed by Western blotting and detection with rabbit anti-c-Src, anti-Lck, and anti-Lyn antibodies. G, 3T3/MUC1 cells (1 × 10^6 cells) were stimulated with sSiglec-9 in the presence (lane b) or absence (lane a) of PP2 (10 μM) for 20 min, and β-catenin co-immunoprecipitated with MUC1 was detected as described in A. H, the intensity of β-catenin in H was estimated as described in D, and the level of β-catenin relative to that of MUC1-CD was compared. The value obtained in the experiment in which 3T3/MUC1 cells were treated with sSiglec-9 in the absence of PP2 (10 μM) for 20 min was taken as 1 (mean ± S.D., n = 3; *p < 0.01).

To further confirm the movement of β-catenin, subcellular fractionation of 3T3/MUC1 cells was performed. The purity of the nuclear fraction was confirmed by the presence of histone 2B and the absence of cytoplasmic IκB-α protein (Fig. 6D, lanes a and b). MUC1-CD and β-catenin were detected in the nuclear fraction prepared from 3T3/MUC1 cells treated with sSiglec-9 (Fig. 6E, lane b), but not in that from the nontreated cells (Fig. 6E, lane a). Co-localization of MUC1-CD and β-catenin (arrows) was also demonstrated immunocytochemically in the nucleus of 3T3/MUC1 cells treated with sSiglec-9 but not in those of 3T3/MUC1 cells not treated with sSiglec-9 (Fig. 6F). These results indicate that β-catenin was transported from the cytoplasm to the nucleus with MUC1-CD after ligation of sSiglec-9 with MUC1. We next studied the effect of sSiglec-9 on the proliferation of 3T3/MUC1 cells. Cells were treated with sSiglec9 for 20 min in serum-free medium and then cultured in RPMI 1640 medium and 10% FCS for 72 h. The level of c-myc was elevated by the ligation of sSiglec-9 with MUC1 (Fig. 6G), this being consistent with enhanced growth in sSiglec-9-treated 3T3/MUC1 cells (Fig. 6H).

**DISCUSSION**

It has been shown that MUC1 is aberrantly overexpressed in a wide range of tumors and is associated with a poor prognosis (27). With transformation and loss of polarity, MUC1 is expressed throughout the entire cell membrane of tumor cells, allowing it to interact with receptors or ligands that are normally restricted to the lateral and basal borders. In the last decade, the function of MUC1-CD has been studied extensively.

affected in 3T3/mock cells (Fig. 6, A and B). These results suggest that ligation of sSiglec-9 with MUC1 enhanced the recruitment of β-catenin and thereby inhibited the phosphorylation of β-catenin by GSK-3β, maybe leading to stabilization of β-catenin and contribution to the malignant phenotype. The nuclear transport of β-catenin after its recruitment to MUC1-CD was examined immunocytochemically and biochemically. 3T3/MUC1 cells were treated with or without sSiglec-9 for 1 h and then observed after staining with propidium iodide and fluorescein-labeled anti-β-catenin antibodies. In the 3T3/ MUC1 cells treated without sSiglec-9, most β-catenin seemed to be distributed in the cytoplasm. However, in the cells treated with sSiglec-9, accumulation of β-catenin was observed in the nucleus (Fig. 6C, arrows). 3T3/MUC1 cells containing β-catenin in the nucleus were counted and were revealed to be increased approximately 3-fold on the treatment with sSiglec-9 (data not shown).
However, studies on signaling through MUC1 following direct ligation of MUC1-ND with an extracellular binding protein have been limited. ICAM-1 has been revealed to be the only direct ligand of the extracellular domain of MUC1, and MUC1 initiates a calcium signal after ligation by ICAM-1 (12, 13). We speculated that mucins readily come into contact with lectins expressed on infiltrating cells in tumor tissues and that lectins could play a role as plausible, physiological ligands for MUC1. Among lectins, the siglec family seem to be potential candidates as counterreceptors for MUC1 because mucins generally possess a variety of O-glycans with terminal sialic acids.

First, we examined the binding of various recombinant siglecs to MUC1. Siglec-9 preferentially bound to MUC1. This may be because Siglec-9 exhibits a relatively wide sugar specificity range including α2–3 and α2–6 sialic acid-linked O-glycans (28, 29).

Siglec-9 is expressed on neutrophils, monocytes/macrophages, and dendritic cells (30, 31). The microenvironment of solid tumors is characterized by a reactive stroma with an abundance of inflammatory mediators and leukocytes. Thus, we examined the distribution of Siglec-9-expressing cells in MUC1-positive tumor tissues. Expectedly, in pancreas, colon, and breast tumor tissues, many Siglec-9–positive cells seem to be associated with MUC1–positive cells (Fig. 2), suggesting that Siglec-9 may actually play a role as a counterreceptor for MUC1.

Next, we tried to examine the recruitment of β-catenin to MUC1-CD through ligation of sSiglec-9 with MUC1-ND expressed on 3T3/MUC1, HCT116/MUC1, and A549 cells, because an important feature of overexpressed MUC1 in tumor cells is its interaction with β-catenin via a peptide motif in MUC1-CD (32, 33), and the level and intracellular distribution of β-catenin play important roles in the morphogenesis and progression of colorectal adenocarcinomas (34). In this experiment, β-catenin was detected in the MUC1-CD immunoprecipitate only when 3T3/MUC1 and HCT116/MUC1 cells were treated with sSiglec-9. In reciprocal experiments, co-immunoprecipitation of MUC1-CD and β-catenin was confirmed (Fig. 4). It has been reported that c-Src physically associates with MUC1, and phosphorylation of MUC1-CD by c-Src increases the formation of MUC1-CD–β-catenin complexes (6, 35).

**FIGURE 5.** MUC1-mediated signaling in 3T3/MUC1 and HCT116/MUC1 cells co-incubated with Siglec-9-expressing cells and effect of neuraminidase treatment of MUC1-expressing cells. A, expression of Siglec-9 in HEK293/mock and HEK293/Siglec-9 cells was analyzed by flow cytometry. B, binding of MAM to the surface of 3T3/MUC1 and HCT116/MUC1 cells was analyzed before (thick solid trace) and after (thin solid trace) neuraminidase treatment with a flow cytometer as described under "Materials and Methods." Control analysis (broken trace) was performed using intact cells without MAM. C, lysates of 3T3/MUC1 and HCT116/MUC1 cells (1 × 10^6 cells) were subjected to SDS-PAGE, followed by Western blotting and detection with mouse anti-MUC1-ND antibodies. D, binding of sSiglec-9 to 3T3/MUC1 and HCT116/MUC1 cells (1 × 10^6 cells) was examined before (thick solid trace) and after (thin solid trace) neuraminidase treatment. Control analysis (broken trace) was performed using intact cells without Siglec-9. E, 3T3/MUC1 and HCT116/MUC1 cells (1 × 10^6 cells) treated with or without neuraminidase were co-incubated with HEK293/Siglec-9 or HEK293/mock cells, and then recruitment of β-catenin to MUC1-CD was examined as described above. F, the intensity of β-catenin in E was estimated as described in Fig. 4D, and the level of β-catenin relative to that of MUC1-CD was compared. The value obtained in the experiment in which 3T3/MUC1 and HCT116/MUC1 cells were stimulated with sSiglec-9 before neuraminidase treatment was taken as 1 (mean ρ ± S.D. (error bars), n = 3, *p < 0.05).
Siglec-9-triggered Recruitment of β-Catenin to MUC1

FIGURE 6. Down-regulation of β-catenin phosphorylation and enhancement of nuclear transport of β-catenin by treatment with sSiglec-9. A, 3T3/MUC1 cells were stimulated with or without sSiglec-9 for 40 min. Phosphorylated β-catenin, β-catenin, and β-actin were detected with mouse anti-phosphorylated β-catenin, anti-β-catenin, and anti-β-actin antibodies, respectively, after SDS-PAGE and Western blotting (IB). B, the intensity of phosphorylated β-catenin in A was estimated as described in Fig. 4D. The level of phosphorylated β-catenin relative to that of β-catenin was compared. The obtained value in the experiment in which 3T3/MUC1 cells were not treated with sSiglec-9 was taken as 1 (mean ± S.D.).

Genetic and molecular cloning of nuclear transport of 3T3/MUC1 cells were not treated with sSiglec-9 was taken as 1 (mean ± S.D.). The conformational change of MUC1 with the ligation of Siglec-9 may stimulate c-Src. The details are currently under investigation.

Brief co-incubation of MUC1-expressing cells with Siglec-9-expressing cells also induced signaling, indicating that the cell-cell interaction through MUC1-Siglec-9 binding could initiate the signaling (Fig. 5). It is generally agreed that most siglecs are masked by endogenous cis ligands. However, they can interact with trans ligands that are structurally easily accessible and carry a high level of appropriately linked sialic acids. MUC1 seems to be one of the most preferential trans ligands for Siglec-9 because MUC1 is an extremely high molecular glycoprotein with high valence due to its tandem repeat and easily accessible to Siglec-9 on the cell surface due to its rod-like structure, which is longer (~250 nm) than typical cell surface adhesion molecules (~28 nm) (36). Furthermore, neuraminidase treatment of MUC1-expressing cells almost completely abolished the effect of Siglec-9 on the recruitment of β-catenin to MUC1-CD, indicating that MUC1-mediated signaling was initiated through the interaction between sialic acid residues expressed on MUC1 and Siglec-9 (Fig. 5).

As in the case of FGF-dependent signaling, recruited β-catenin was transported to the nucleus. This transport of β-catenin was also elevated when 3T3/MUC1 cells were stimulated with sSiglec-9, indicating that the recruitment and nuclear transport of β-catenin do not take place simply on over-expression of MUC1 but are induced by ligation with external ligands. It is also known that GSK-3β phosphorylates β-catenin and thereby targets it for proteosomal degradation (37, 38). Ligation of MUC1 with sSiglec-9 decreased phosphorylated β-catenin, maybe resulting in an increased nuclear level of β-catenin (Fig. 6, A and B). These results are consistent with the fact that MUC1-induced stabilization of β-catenin results in increases in nuclear β-catenin and activation of Wnt target genes. β-Catenin acts as a transcriptional co-activator that...
increases the expression of cell cycle progression genes cyclin-D1 and c-myc, thus leading to the development of diverse tumor malignancies (11). This may be an another MUC1-mediated pathway that promotes tumor progression even though growth factors such as EGF and FGF or their receptors are absent.

In addition, Siglec-9 may bind to various glycoproteins on the cell surface. In fact, Siglec-9 could bind commonly to a number of glycoproteins on the surface of both 3T3/mock and 3T3/MUC1 cells (data not shown). At present, it is uncertain whether or not these membrane glycoproteins are related to Siglec-9-triggered Recruitment of β-Catenin to MUC1 signaling. Mol. Cancer Res. 4, 873–883

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