Siglec-7 mediates nonapoptotic cell death independently of its immunoreceptor tyrosine-based inhibitory motifs in monocytic cell line U937

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Siglec-7, a sialic acid binding immunoglobulin-like lectin, predominantly transduces inhibitory signals through cytosolic immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Here, we report that clustering of Siglec-7 with a specific F(ab′)2 elicited cell death. Interestingly, a truncated Siglec-7 lacking the cytosolic ITIM domain still induced the cell death, suggesting that the ITIMs are not essential for the death signaling. Further analyses of the death signaling revealed that an oxygen radical scavenger, N-acetyl cysteine, completely inhibited the cell death, whereas a pancaspase inhibitor did not. In addition, caspase-3 activation, DNA ladder formation, and nuclear condensation were not detected during the death process, suggesting that the cell death is nonapoptotic. To identify the critical region for the death signaling, we prepared a series of shuffling chimeras between Siglec-7 and Siglec-9, the latter of which did not transduce a death signal. The critical region was mapped to the middle of the membrane-proximal C2-set domain, which contained only six amino acid differences between Siglec-7 and Siglec-9. Point mutation analyses of each of these six amino acids revealed that four of the six amino acids were critical for the death signal. A computer-assisted 3D modeling revealed that these four amino acids were proximally located on the surface of the C2-set domain. In conclusion, Siglec-7 induces nonapoptotic cell death, the signal for which is transduced by an extracellular C2-set domain.

Keywords: cell death/monocytes/natural killer cells/Siglec

Introduction

Natural killer (NK) cells comprise a subset of lymphocytes and play important roles in innate immunity for killing tumor cells and virus-infected cells. Recognition of tumor cells by NK cells involves two types of receptors, namely activating receptors and inhibitory receptors. Inhibitory receptors prevent overactivation of NK cells, thereby preventing them from attacking normal cells and tissues (Sentman et al. 2006). It is generally accepted that MHC class I molecules on target cells are the main inhibitory ligands against the cytotoxicity of NK cells, but additional molecules, including cell surface glycans, are involved in cell recognition.

Siglec-7, a member of the Siglec family, is an inhibitory receptor expressed on NK cells and monocytes (Falco et al. 1999; Nicoll et al. 1999). Using multivalent carbohydrate ligands, we previously found that Siglec-7 preferentially binds to α2,8-disialyl and branched α2,6-sialyl carbohydrate structures (Yamaji et al. 2002). α2,8-disialyl carbohydrates are tumor-associated antigens, and typically found on human melanoma cells. It has been speculated that cancer cells such as melanoma cells express α2,8-disialyl carbohydrates to activate Siglec-7 and consequently inhibit NK cell functions. Siglec-7 shows the highest homology to Siglec-9 (83% identity in the amino acid sequences) and is expressed strongly in NK cells and weakly in monocytes (Nicoll et al. 1999). We previously carried out several shuffling experiments of these two S Igles and identified the carbohydrate-binding domain (Yamaji et al. 2002).

Immunoreceptor tyrosine-based inhibitory motif (ITIM) domains transduce the intracellular signaling of Siglecs via tyrosine phosphorylation to reduce or inhibit the activating signals of immune cells, including NK cells. We previously reported that the ITIM domain of Siglec-7 recruits the protein tyrosine phosphatase Src homology-2 (SH2) domain-containing protein-tyrosine phosphatase-1 (SHP-1) (Yamaji et al. 2005).

Several Siglec molecules have recently been reported to be involved in the cell death of lymphocytes (Nakura et al. 2003; von Gunten et al. 2005). Nakura et al. (2003) reported that cross-linking of Siglec-8 induces apoptosis of human eosinophils, and that this apoptosis is mediated by caspase-3. Meanwhile, von Gunten et al. (2005) reported that Siglec-9 transduces apoptotic and nonapoptotic cell death of neutrophils. They found that the nonapoptotic cell death is dependent on reactive oxygen species. Thus, an accumulating amount of evidence indicates that the Siglec family members mediate the cell death of blood lymphocytes in a cell-type-specific manner (Nakura et al. 2003; von Gunten et al. 2005).

In the present study, we found that Siglec-7 mediates the cell death of U937 cells. Interestingly, the cell death was completely inhibited by N-acetyl cysteine (NAC), a scavenger of reactive oxygen, suggesting that the cell death occurs via...
oxidative stress. Since Siglec-7 is highly homologous to Siglec-9, with 83% identity in their amino acid sequences, and Siglec-9 did not show cell death in the U937 cells, we used a domain-swapping strategy, in which portions of Siglec-7 were replaced with the corresponding amino acids of Siglec-9. Chimeric analyses of the Siglec-7 mutants revealed that part of the extracellular membrane-proximal C2-set domain strongly contributes to the cell death. Furthermore, we found that four amino acid residues in the C2-2 region are critical for the cell death. These findings provide insights into Siglec-7-mediated intracellular signaling and may contribute to research into antitumor and antivirus treatments mediated by NK cells.

Results

Ligation of Siglec-7 by a monoclonal antibody induces cell death—U937 cells were transfected with a Siglec-7 cDNA. The established cell line, designated WT7, exhibited >10-fold higher expression of Siglec-7 than the parental U937 cells (Figure 1A). Using this cell line, we examined whether treatment with the F(ab′)2 fragment of the anti-Siglec-7 monoclonal antibody 13-3-D was able to elicit cell death (Figure 1B and C). We considered that this system would be a good model for analyzing the domain functions of Siglec-7. Treatment of the cells with this specific antibody increased the cell death to 30–50%.

A control F(ab′)2 fragment of an irrelevant antibody exerted a subtle effect on the cell death under our experimental conditions. Although the parental U937 cells expressed low levels of endogenous Siglec-7, anti-Siglec-7 F(ab′)2 treatment did not increase their cell death, probably because the death signal was not sufficiently strong to induce cell death (Figure 1). The cell death of Siglec-7-expressing cells was time- and dose-dependent for incubation with the F(ab′)2 fragment (Figure S1B). Longer incubation of the cells produced a slight increase in the ratio of annexin V-positive cells, probably because the small amounts of medium in the assay system were not sufficient to allow cell survival for longer periods of time.

Next, we tried to characterize the cell death by several experiments. A scavenger for reactive oxygen, N-acetyl cysteine (NAC), completely inhibited the cell death (Figure 2A). In contrast, a pancaspase inhibitor, Z-VAD-FMK, did not inhibit the cell death, although it did inhibit TNF-α-induced cell death (Figure S2A). In addition, we did not detect DNA ladder formation during the cell death process although it was detected in the TNF-α-induced cell death (Figure S2B). Taken together, these findings suggest that oxygen radicals, rather than a caspase-mediated cascade, contributed to the cell death. Electron microscopy revealed that ligation of Siglec-7 induced a characteristic vacuolization of the rough endoplasmic reticulum (rER), which was totally abolished by NAC treatment (Figure 2B). According to a quantitative analysis, approximately 55% of the cells contained more than five swollen rER profiles in the antibody-treated condition, while this percentage was significantly decreased to <10% in the presence of NAC (Figure 2C). Pyknotic nuclei, which represent a typical feature of apoptosis, were hardly detected stochastically, thereby supporting the notion that the cell death was not apoptotic.

Subsequently, we swapped domains between Siglec-7 and Siglec-9 to identify the domains contributing to the cell death. Siglec-9 is highly homologous to Siglec-7 (83% homology), but exhibited a different phenotype in the death-inducing activity as evaluated by the following experiments. The expression levels of Siglec-9 and the swapped chimeras were in a similar range to those of Siglec-7, as determined using an anti-Siglec-9 antibody. No cell death of Siglec-9-expressing cells was elicited by anti-Siglec-9 F(ab′)2 treatment (Figure 3A, WT9). This finding...
ITIM-independent cell death signaling by Siglec-7

Fig. 2. NAC completely inhibits the anti-Siglec-7 antibody-induced cell death. (A) Cell death assays of U937 cells (left) and wild-type Siglec-7-expressing WT7 cells (right) with or without NAC treatment. (B) Electron microscopic observations of anti-Siglec-7 antibody-treated cells with (right) or without (left) NAC treatment. A higher magnification image of the boxed is shown in the inset. Many profiles of swollen rER, some of which are indicated by arrows, are present in the cells treated with the anti-Siglec-7 antibody (arrows). N, nucleus. Bars: 10 μm, and 0.5 μm in the inset. (C) Quantification of the electron microscopic data shown in B. Cells were categorized into three groups based on the numbers of vacuolar profiles of swollen rER per cell as followed: open bars, no swollen rER; gray bars, 0 < swollen rER ≤ 5; filled bars, >5 swollen rER. Thirty cells per grid were analyzed and counted on the electron micrograph. The data represent the means and standard deviations of the percentage of each category calculated from three distinct grids of each sample. The data were compared using the Aspin–Welch test of significance.

was not caused by a lack of the death-inducing activity of the anti-Siglec-7 antibody, because the antibody induced cell death for a chimera consisted of the V-set domain of Siglec-9 and the remaining portion of Siglec-7 (V-9) (Figure 3A). These findings further suggested that the V-set domain of Siglec-7 was not important for the cell death and that the remaining portion transduced the signal. To examine the involvement of the ITIMs in the signaling, we deleted the cytoplasmic portion of Siglec-7 (7Δcytosol) and expressed the mutant in U937 cells. Surprisingly, 7Δcytosol elicited the cell death at the same level as the wild-type Siglec-7 (Figure 3B). These results suggested that the two C2-set domains and the upper part of the transmembrane region were critical for the cell death.

To narrow down the region responsible for the cell death, we created additional chimeric mutants, in which each C2-set domain of 7Δcytosol was replaced with the corresponding C2-set domain of Siglec-9 (Figure 3C). Replacement of the membrane-proximal C2-set domain (C2-2) abolished the cell death activity, while replacement of the distal C2-set domain (C2-1) did not, indicating that the proximal C2-2 domain was important for the cell death. The C2-2 domain was divided into three portions, termed the C2-2a, C2-2b, and C2-2c regions. When the C2-2b region was replaced with the corresponding region of Siglec-9...
(C2-b-9), the cell death was completely abolished. In contrast, replacement of the C2-2a region had no effect and replacement of the C2-2c region and the upper part of the transmembrane region had little effect on the cell death. These results suggested that the C2-2b region contributed most strongly to the cell death. A partial effect of the upper transmembrane region and cytosolic domain was observed in one mutant (extracellular-9). Since the cytosolic domain did not completely contribute to the cell death, the upper transmembrane domain had a slight effect on the cell death.

There were six amino acids that differed between the C2-2b regions of Siglec-7 and Siglec-9 (Figure 4A, alignment). Therefore, we attempted to prepare single amino acid mutants of these six amino acids using the 7Δcytosol cDNA as a parental construct. Five of the six possible mutants were established as stable cell lines and subjected to the cell death assay. Four of these five mutants (W288L, T289S, S292G, and L304G) showed marked decreases in the cell death activity (Figure 4A), suggesting that these four residues were the most critical for inducing the cell death.

To visualize the possible spatial locations of the four amino acids in the C2-2 domain, we constructed a 3D model of the C2-2 domain (Figure 4B). A homology search using the Smith–Waterman algorithm was performed using SSearch (Smith and Waterman 1981; Pearson 1991) to identify sequences that were homologous with the human Siglec-7 C2-2 domain (supplementary Figure S3B). The second immunoglobulin domain of human paladin (PDB code 2dm3) was selected as a template among those with low E values, mostly because the positions of the cysteine residues were conserved between the domains of these two proteins. In this model, the four critical amino acids (red) were located closely together at the surface of the molecule, despite the fact that Leu304 was 16-amino-acid residues away from Trp288 in the primary sequence. Gln309, which did not appear to be involved in the cell death signaling, was located distally from the cluster of four amino acids in the 3D model.

Discussion

Siglecs, sialic acid-binding immunoglobulin-like lectins, are cell-surface proteins that recognize sialic acid-containing carbohydrate chains of glycoconjugates, such as glycolipids and glycoproteins, in cis or trans manners. To date, 13 species of Siglec molecules have been identified in humans (for a review, see Crocker et al. (2007)). They are expressed in various tissues and are thought to function in the innate and adaptive immune systems through carbohydrate recognition. Siglec-7, one of the members of the family, is expressed in NK cells and monocytes, which attract the attention of researchers in innate immunity.

In the present study, we have demonstrated that the cell death of U937 cells is elicited by ligation of Siglec-7 with the F(ab')₂ fragment of an anti-Siglec-7 antibody, and identified critical amino acids in an extracellular domain of Siglec-7. Although the cell death was observed in a cultured monocytic cell line, our findings provide insights into the signaling mechanisms of Siglec-7 in NK cells and monocytes in vivo. The findings for our findings provide insights into the signaling mechanisms of Siglec-7 in NK cells and monocytes in vivo. The findings for the 7Δcytosol mutant lacking the cytosolic domain revealed that the ITIMs in the cytosolic domain were not essential for the cell death response. The cell death was induced by even 1 μg/mL of the F(ab')₂ fragment, which was prepared from a rat monoclonal antibody, suggesting that there is little possibility that trace amounts of the intact antibody were responsible for the effect. SDS–PAGE evaluation of the antibody supported our conclusion that impurity of the antibody preparation was not responsible for the induction of the cell death (supplementary Figure S1A). Clustering of Siglecs with specific antibodies has already been reported to induce cell death (Nutku et al. 2003; von Gunten et al. 2005, 2006). Our data focusing on shuffling experiments have provided structural and biochemical insights into the functions of Siglec-7 in cell death.

Fig. 4. (A) Analyses of point mutants in the C2-2b domain. The C2-2b domain of Siglec-7 contains six amino acid differences from Siglec-9. We created six amino acid point mutants of the Siglec-7 cDNA for transfection of U937 cells and established five stable mutants. Cell death assays of the mutant cell lines were carried out by Annexin V/PI staining. The experiments were carried out at least twice in triplicate. Standard deviations were calculated using the value for the specific antibody-treated cells. Two or three cell lines were tested for each mutation and confirmed that the results were not clone dependent. Partial sequences of the C2-2b regions of Siglec-7 and Siglec-9 are shown below the graph. The numbers above the sequences refer to the amino acid positions in Siglec-7. The different amino acids between the sequences are boxed. (B) Computer-assisted models of the C2-2 domains of Siglec-7. The space filling model of Siglec-7 (gray) was calculated and displayed with the MODELLER version 9.4 software. The different amino acids between the C2-2b region of Siglec-7 and that of Siglec-9 are red. The model reveals the close locations of the four amino acids (blue labeled) that are critical for the cell death activity.
Our system involving overexpression transfectants and antibody stimulation to induce cell death may lead to arguments about artificial experiments that are not relevant to NK cells or monocytes expressing Siglec-7 endogenously. However, several lines of evidence have been reported that support the results of the present study as follows. Carbohydrates, especially glycosphingolipids, are clustered with cholesterol and signaling molecules on the surface of the plasma membrane as lipid rafts, and this clustering is important for signal transduction (Simons and Ikonen 1997). GD3, a glycolipid ligand for Siglec-7, is also contained in lipid rafts (Kasahara et al. 1997). Therefore, it is conceivable that the clustering of Siglec-7 with the antibody mimics the clustering of these molecules with carbohydrate ligands on target cells in a trans-acting manner, thereby leading to activation of signaling molecules. We tried to use polyvalent carbohydrate ligands to stimulate Siglec-7-transfected cells, but found that they did not cause cell death, probably because these ligands were less competitive than the endogenous ligands on the cells themselves (data not shown). Next, we examined K562 cells expressing a Siglec-7 ligand. However, these cells also did not elicit any cell death of transfected U937 cells. Finally, we found that antibody-mediated clustering of Siglec-7 was effective for inducing cell death. Therefore, the system is quite useful for gaining insights into signal transduction mechanisms.

It is possible that expression of Siglec-7 itself in the cells altered the proliferation rate. Measurement of the proliferation rates of the parental U937 cells and cells transfected with Siglec-7 lacking the cytosolic region (Δcytosol) revealed that the two types of cells showed similar proliferation rates. Specifically, the proliferation rates of U937 and 7ΔCytosol cells at 2 × 10^5 cells/mL were 381.7 ± 78.0% and 345.0 ± 72.8% after 27.5 h, respectively.

Previous studies have shown that CD33-expressing leukemia cells exhibit reduced proliferation after treatment with an anti-CD33 antibody (Balaian and Ball 2001, 2004, 2005; Balaian et al. 2003), which is mediated by Syk protein signaling. We need to try to determine whether tyrosine phosphorylation of Syk is involved in the cell death. On the other hand, Vitale et al. (1999) reported that the monoclonal antibody against p75/AIRM-1 (Siglec-7) inhibits proliferation of myelomonocytic cell precursors and chronic myeloid leukemias. Our counting cell revealed that the anti-Siglec-7 antibody did not change the number of U937 cells (data not shown).

Domain shuffling between Siglec-7 and Siglec-9 revealed that the middle portion of the C2-2 domain (C2-2b) was the most critical region for transducing the cell death signal. Single amino acid swaps in part of the C2-2b domain identified four particular amino acid residues that were important for the signal transduction. We need to discuss the possibility of clonal selection of the Siglec stable transfectants. Multiple clones for each point mutant were selected and determined for their expression levels and normalized cell death values (supplementary Table SI). Although clones with high expression of Siglec-7 tended to show more cell death while those with low expression tended to exhibit less cell death, the results were consistent with our conclusion that the four amino acids in the C2-2b region are critical for the cell death. To obtain sufficient evidence that the C2-2 domain is the critical portion for the cell death, we tried to obtain a mutant expressing Siglec-9 in which the C2-2 region was substituted by that of Siglec-7. However, the mutant could not be isolated, probably because of its cell lethality. Further experiments, such as those using inducible vectors, may be needed to clarify this point.

Computer modeling of the 3D structure of the C2-2 domain predicted that the four amino acids were proximally located to one another, suggesting that these amino acids would be involved in interactions with other adjacent molecules to transduce the death signal. The qualities of the resultant protein structures were checked using the Procheck program (Laskowski et al. 1993), which gives Ramachandran plots and a quantitative distribution of the geometric parameters within the allowed conformational space. The percentages of residues in the most favored, additionally allowed, generously allowed, and disallowed positions were 90.3%, 6.9%, 2.8%, and 0.0%, respectively. Indeed, the model is considered to show the structure of the primary C–C bonds precisely, whereas the side chains of the amino acids may be speculative. X-ray crystallography or NMR analysis may produce more precise information about these side chains in further experiments.

Four possible arguments regarding the cell death mediated through the membrane-proximal C2 region arise as follows. First, interactions with membrane-spanning proteins may transduce signals through the C2-2 domain. Appropriate candidates could be transmembrane molecules with a signaling function, since Siglec-7 lacking the cytosolic domain still transduced the death signal and a very small region of the extracellular C2-2 domain was important for the signaling. It still remains possible that a candidate for the interacting molecule may be endogenous Siglec-7 with ITIMs, or that co-ligation of endogenous Siglec-7 with Δcytosol, induced the phosphorylation of endogenous ITIMs to transduce the death signal. However, this scenario is fairly unlikely, because ligation of endogenous Siglec-7 alone did not induce the cell death, and ligation of the V-9 mutant with the anti-Siglec-9 F(ab′)2 still induced the cell death although co-ligation of endogenous Siglec-7 was not expected (Figure 3A). Some Siglecs are already known to interact with membrane-spanning signaling molecules. For example, Siglec-15 associates with DAP-12 and DAP-10, in which a lysine residue in the Siglec-15 transmembrane region interacts with an Asp residue in DAP-12 or DAP-10 (Angata et al. 2007). Siglec-H also interacts with DAP-12 and modulates the secretion of type I interferon by interferon-producing cells (Blasius et al. 2006; Blasius and Colonna 2006). Likewise, Siglec-14 interacts with DAP-12 through an Arg residue in its transmembrane domain. It is therefore possible, although less likely, that the DAP family proteins interact with Siglec-7, which does not have basic amino acids in its transmembrane domain. Indeed, immunoprecipitation analyses using an anti-Siglec-7 antibody revealed that no DAP proteins were detected in precipitates from stimulated U937-WT7 cells (data not shown). To identify other possible molecules that interact with Siglec-7, immunoprecipitation analyses using an anti-Siglec-7 antibody are in progress in our laboratory. Second, the conformation of Siglec-7 may be changed by the mutations and affect the ITIM-dependent signaling pathway. This is less likely because even the mutants without the ITIM signaling region still induced the cell death. Third, modification of carbohydrates attached to the molecule may be involved in Siglec-7 functions. There are eight potential N-glycosylation sites in Siglec-7 (Nicoll et al. 1999) and Western blotting analysis of the protein expressed in U937 cells showed that the molecules were highly glycosylated. Further analyses are required to solve this issue. Although there have not
been any reports that carbohydrates attached to the molecule itself are involved in Siglec-7 functions, including ligand binding (Freeman et al. 2001), we observed variations in the molecular weight in Western blotting analyses, probably owing to glycosylation. Fourth, incorporation of the molecules into lipid rafts and micro domains may be altered by the mutations. The carbohydrates on a membrane protein or a conformational change could alter the incorporation of the protein into micro domains such as lipid rafts (Dr. Mie Yokoyama, Tokyo Medical and Dental University, Tokyo, Japan, personal communication). These attractive hypotheses should be examined in further studies.

Ligation of Siglec-7 induced “non-apoptotic cell death,” in which no caspase activation or DNA ladder formation was observed. Morphological analyses of the cells further revealed no indications of typical apoptosis such as nuclear condensation. Instead, NAC treatment prevented the cell death, indicating that reactive oxygen species are involved in the downstream of the Siglec-7-mediated death signal. We are currently trying to elucidate the downstream signaling for the cell death, which would be beneficial for understanding the regulatory mechanism for Siglec-7 functions.

Although our system using U937 cells is good for observing the effects of Siglec-7 cell signaling and suitable for Siglec-7 manipulation, it is very distant from physiological conditions. Therefore, we carried an experiment using peripheral monocytes to clarify whether they showed cell death after treatment with the anti-Siglec-7 antibody. Analysis of peripheral blood monocytes isolated from human blood using Ficoll gradient-density centrifugation and a MACS system revealed that the antibody had no significant effects on cell death (supplementary Figure S4). These cells probably require certain cytokines to enter a state of cell activation or proliferation because such cells are easily susceptible to cell death. On the other hand, it is tempting to speculate that glycan ligands of Siglec-7, such as α2,8-disialyl residues (Yamaji et al. 2002) and a melanoma antigen (Nara et al. 1994), can suppress NK cell functions in vivo. If this is the case, this process could represent a new therapeutic target. For instance, developing chemicals that can modulate the C2 domain of Siglec-7 may be beneficial for understanding the regulatory mechanism for Siglec-7 functions.

Material and methods

Cell culture

U937 cells and their transfectants were cultured in the RPMI1640 medium (Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS; JRH Bioscience, KS, USA) in a 5% CO₂ atmosphere in the absence or presence of 0.5 mg/mL of G418 (Gibco), respectively.

Monoclonal antibodies and preparations of their F(ab’)_2 fragments

A rat anti-Siglec-7 monoclonal antibody (13-3-D) and a rat anti-Siglec-9 monoclonal antibody (1-3-A) were established in previous studies (Miyazaki et al. 2004; Yamaji et al. 2005). Epitope mapping analyses revealed that the 13-3-D and 1-3-A antibodies reacted with the V-set domains of Siglec-7 and Siglec-9, respectively. The F(ab’)_2 fragments of these antibodies were prepared as described previously (Yamaji et al. 2005).

Establishment of stable cell lines expressing Siglecs and their mutants

Siglec cDNAs were cloned into an expression vector, pCXN2, containing a CAGi promoter, which consists of a CMV intermediate early enhancer and a modified chicken β-actin promoter (Niwa et al. 1991). Siglec mutant cDNAs were constructed by PCR-based mutagenesis and subcloned into pCXN2. An aliquot (10 μg) of each plasmid was linearized by SspI digestion and transfected into 1 × 10⁷ U937 cells undergoing log-phase growth. Each linearized DNA was transfected by electroporation using a GenePulser (Bio-Rad, CA, USA) at 300 V and 960 μFD or a GenePulser II (Bio-Rad) at 300 V and 975 μFD in 250 μL of serum-free RPMI1640 in a 0.4-cm cuvette. The transfected cells were cultured in 10 mL of RPMI1640 containing 10% FBS without antibiotics for 2–5 days, prior to the addition of G418 to a final concentration of 0.5 mg/mL. G418-resistant cells emerged after 1–3 months. Selected cells were diluted to a limiting concentration (0.5 cells/well) and seeded in the wells of 96-well plates for cloning. The cloned cells were labeled with anti-Siglec-7 or anti-Siglec-9 antibodies for fluorescence-activated cell sorting (FACS) analyses to examine the expression levels of the Siglecs. For cell death assays, we selected clones that showed similar levels of expression to cells expressing the 7∆cytosol clone, which was determined by the mean fluorescence value in the FACS analysis as described below.

FACS analysis of the expression levels in transfectants

For expression analysis, we used an anti-human Siglec-7 antibody (R&D Systems Inc., MN, USA) and an anti-human Siglec-9 antibody (R&D Systems Inc.) that recognized the extracellular domains of Siglec-7 and Siglec-9, respectively. Cells expressing Siglecs were incubated with the anti-Siglec-7 antibody (5 μg/mL) or anti-Siglec-9 antibody (12.5 μg/mL). Control cells were incubated with goat anti-rat IgG. Subsequently, the cells were incubated with an Alexa Fluor 488-conjugated donkey anti-goat IgG (H+L) secondary antibody (Molecular Probes, OR, USA). The labeled cells were analyzed using a FACSCalibur and the CellQuestPro software (BD Bioscience, CA, USA). The levels of expression were determined as the mean fluorescence values from the obtained histograms.

Cell death assay

The percentages of dead cells were evaluated by Annexin V staining as follows. At 4 days before the assay, the cells were centrifuged for medium exchange and resuspended in the fresh medium (RPMI1640 supplemented with 10% FBS) to 6 × 10⁵ cells/mL. On the day before the assay, the cells were diluted to 8 × 10⁵ cells/mL with the fresh medium to maintain them in the growth phase. On the day of the assay, the cells were washed once with phosphate-buffered saline (PBS) and resuspended in the fresh medium. After a further centrifugation, aliquots (50 μL/well) of the cells (4 × 10⁵ cells/mL) were seeded in 96-well plates (final density, 2 × 10⁵ cells/well) and equilibrated for 30 min in a CO₂ incubator. Next, 5 μL of F(ab’)_2 fragment (200 μg/mL) was added to each well, followed by the addition of 45 μL of medium. An Annexin V-FITC
Detection of dying cells in combination with staining with propidium iodide (PI). After 24 h of antibody stimulation, the cells were centrifuged, resuspended in 50 μL of the kit’s binding buffer containing 25 μg/mL of Annexin-V-FITC and 250 μg/mL of PI, and gently mixed by vortexing. After incubation on ice for 15 min, the cells were resuspended by tapping and transferred to new tubes containing 200 μL of binding buffer. FACS analyses were typically carried out with the following settings: SSC, 362 V; FL1, 450 V; FL3, 535 V depending on the laser power. All assays were performed within 30 min after the final centrifugation to avoid non-specific cellular damage. The control antibody for the cell death assays was an F(ab')2 fraction of rat IgG (Jackson ImmunoResearch, PA). Due to the limited number of samples in an experiment, the normalized activity was calculated to compare the results of different experiments as follows: if the percentage of cell death was R, the normalized activity (%) was calculated by (R\text{sample}, \text{specific antibody} - R\text{WT7 or 7\text{cytlosol}, specific antibody}) / (R\text{WT7 or 7\text{cytlosol}, control antibody}). The data before normalization were shown in Supplementary Figure S3.

Electron microscopy

Cells were fixed with 2% paraformaldehyde–2% glutaraldehyde in the 0.1 M phosphate buffer. They were post-fixed with 1% OsO4, and then embedded in Epon812 and sectioned as previously described (Waguri et al. 1999).

Computer-assisted modeling of 3D structures of Siglecs

We used the MODELLER version 9.4 software (http://salilab.org/modeller/) (Sali and Blundell 1993; Fiser et al. 2000; Marti-Renom et al. 2000; Eswar et al. 2006) to create a model of the C2-2 domain. The second immunoglobulin domain of human palladin (PDB code 2dm3) was used as a template.

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Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

Abbreviations

7Δcytlosol, Siglec-7 lacking the cytosolic region; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; ITIM, immunoreceptor tyrosine-based inhibitory motif; NK cell, natural killer cell; NAC, N-acetyl cysteine; PBS, phosphate-buffered saline; PI, propidium iodide; rER, rough endoplasmic reticulum; WT7, wild-type Siglec-7; WT9, wild-type Siglec-9.

References


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