A Novel Function of Siglec-9 A391C Polymorphism on T Cell Receptor Signaling

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Key Words

Siglec-9 • Homology structure model • Red blood cell binding • T cell receptor-mediated signaling • A391C polymorphism

Abstract

Background: Sialic-acid-binding immunoglobulin-like lectins (Siglecs) are the best-characterized immunoglobulin-type lectins. There is a growing amount of data linking Siglec and autoimmune diseases. The recently identified Siglec-9 inhibits T cell receptor (TCR)-mediated signaling which has been demonstrated by site-directed mutagenesis. In human Siglec-9, at least 8 nonsynonymous SNPs have been detected without functional studies. This study examined the SNP(s) related to TCR-mediated signaling. **Methods:** Since the functions of Siglecs are modulated by their interaction with sialic-acid-containing carbohydrate groups, a molecular modeling analysis of carbohydrate binding interactions and an RBC binding analysis were performed using the 8

SNPs. The TCR-mediated signaling was analyzed with the downstream signaling molecules ZAP-70 and IL-2. **Results:** This study revealed that an A391C polymorphism is the only mutant related to the binding. Jurkat T cells transfected with the A391C mutant reduced the inhibition of ZAP-70 phosphorylation and IL-2 production compared to cells transfected with the wild type. **Conclusions:** Siglec-9 A391C was the only polymorphism related to TCR-mediated signaling in human Siglec-9, resulting in less inhibition compared to the wild type.

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Introduction

Sialic-acid-binding immunoglobulin-like lectins (Siglecs) are thoroughly characterized immunoglobulin (Ig)-type lectins [1]. They are type I membrane proteins containing multiple extracellular Ig-like domains that recognize different types and linkages of sialic acid [2]. The

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1018–2438/11/1542–0111\$38.00/0

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CD33-related Siglecs seem to be evolving rapidly, and there are 9 CD33-related Siglecs and 1 Siglec-like protein in humans. An important function of the CD33-related Siglecs is their ability to regulate cell growth and survival, either by inhibition of proliferation or induction of apoptosis. Most CD33-related Siglecs have 1 or more immunoreceptor tyrosine-based inhibitory motifs in their cytoplasm tail [2] for the recruitment of Src homology region 2 domain-containing phosphatase (SHP)-1 and SHP-2 and for inhibitory signaling functions [3–5]. The structure of the CD33-related Siglec-7 has been determined, and it provides a structural prototype for the other members of this family including Siglec-9 [6].

Siglecs have a cell type-specific expression, and the recent identification of Siglec-9 is strongly expressed as Siglec-7 on neutrophils and less so on monocytes, NK cells [5], and B and T cells [12, 13]. In neutrophils, the antibody-induced ligation of Siglec-9 induces cell death [7], which has been shown to be responsible for the cytokine-dependent cytotoxic effect of intravenous Ig on inflammatory cells including neutrophils [8]. Intravenous Ig has been used for the treatment of autoimmune and systemic inflammatory diseases [9–11]. Siglec-9 in macrophages has induced anti-inflammatory cytokine and interleukin (IL)-10 production [14] to suppress excess proinflammatory cytokine production which is harmful to the host [15]. In T cells, it regulates T cell receptor (TCR) activation by the recruitment of SHP-1, resulting in reduced transcription of the nuclear factor of activated T cell (NFAT)-mediated cytokine gene as Siglec-7 [16]. In fact, human-specific loss of T cell Siglec expression has been shown to be associated with T cell hyperactivity, whereas transfection of Siglec-7 and Siglec-9 into the Jurkat T cell line inhibits TCR-mediated signaling [17]. Thus, Siglec-9 is emerging as an important regulator of the immune system.

The inhibitory functions of Siglec-9 as well as Siglec-7 have been demonstrated by site-directed mutagenesis [5]. Studies on Siglec polymorphisms among humans have been proposed on the basis of a growing number of data linking inhibitory receptor polymorphisms and autoimmune diseases [18]. In human Siglec-9, at least 8 non-synonymous SNPs, including rs1697545, rs2075803, rs200658, rs16988910, rs273687, rs2258983, rs273688, and rs273690 (dbSNP accession No.), have been detected. However, there have been no functional studies on any of them. The functions of Siglecs are modulated by their interaction with sialic-acid-containing carbohydrate groups of glycoproteins and glycolipids as ligands. Particularly, the C-C' loop in the sugar-binding domain

plays a major role in determining the binding specificities of Siglec-7 and Siglec-9 [19].

In this study, we examined carbohydrate binding affinity with a molecular modeling analysis [6, 20] and an RBC binding assay using the 8 mutants. Both results showed that the changed amino acid residue in the A391C polymorphism (dbSNP accession No. rs16988910) could be the only mutant affected in terms of binding affinity. In the functional study of the mutant, an increased ZAP-70 phosphorylation in the variant compared with the wild type was confirmed, along with a reduced inhibition of NFAT-mediated cytokine gene transcription such as IL-2 production in transfected Jurkat cells.

Materials and Methods

Analysis of the Siglec-9 Homology Model Structure

To investigate a possible relationship between the A391C polymorphisms identified in Siglec-9, we used a 3-dimensional (3D) model of the Siglec-9 protein constructed based on the crystal structure of Siglec-7. The sequence alignment of Siglec-9 and Siglec-7 was generated using FASTA software (<http://www.ebi.ac.uk/fasta33>). A 3D model structure of Siglec-9 was built using the HOMOLGY module of Insight II (Accelrys Software, Inc., San Diego, Calif., USA) and was further refined by using the Discover version 2.98 program of Insight II. The 3D model structure of Siglec-9 was visualized with PYMOL (<http://www.pymol.org>).

The Siglec-9 and carbohydrate docking model was generated by superposing the Siglec-7 and carbohydrate complex and by using an X-ray structure with PDB code 1RVT.

Cell Culture

Jurkat cells (No. 40152; KCLB, Seoul, South Korea) were cultured in RPMI 1640 (WelGENE, Inc., Daegu, South Korea) supplemented with 10% heat-inactivated fetal bovine serum (WelGENE), 100 units/ml of penicillin and 100 units/ml of streptomycin (Invitrogen, Carlsbad, Calif., USA). COS-7 cells (No. 21651; KCLB) were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml of penicillin and 100 units/ml of streptomycin.

Expression Constructs

The entire coding sequence of Siglec-9 was amplified by PCR using a Siglec-9 clone purchased from the 21C Human Gene Bank, Genome Research Center, KRIBB (South Korea), as a template. The PCR product was inserted into the pcDNA3 vector (Invitrogen). Eight mutant constructs of Siglec-9, including rs1697545 (Y45*), rs2075803 (K100E), rs200658 (S125N), rs16988910 (K131Q), rs273687 (N147K), rs2258983 (A315E), rs273688 (A316D), and rs273690 (V349A), were prepared by inverse PCR.

Transient Expression of Siglec-9 and the Mutants

After having been washed with RPMI 1640 and resuspended in a Nucleofector Kit V (Amaxa, Cologne, Germany), the Jurkat cells were nucleofected according to the manufacturer's guidelines. Briefly, a 100- μ l suspension of 4×10^6 cells mixed with

Fig. 1. Model structure analysis of Siglec-9. **a** In the 3D structure analysis of Siglec-9, the red color denotes SNP sites. K100E, S125N and K131Q are located in the carbohydrate recognition domain and N147K is positioned in the Ig-like domain. **b** Binding model of the carbohydrate for the wild type (left) and the K131Q mutant (right). The dotted lines denote hydrogen bond interactions between the carbohydrate and Siglec-9.

7 μ g of each plasmid DNA was transferred to the provided cuvette and nucleofected with a Nucleofector apparatus on the S-018 program setting. The cells were immediately transferred into wells in 60-mm dishes, which contained culture medium prewarmed to 37°C.

The COS-7 cells were transfected with the vectors using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Briefly, 4 μ l per well of Lipofectamine 2000 and 1.6 μ g of plasmid DNA were diluted in Optimem (Invitrogen). Five hours after the transfection, all cells were cultured for another 48 h and flow cytometry analysis was then performed.

Flow Cytometry Analysis

Surface expressions of the wild-type and mutant cells were evaluated by flow cytometry after direct staining with FITC-labeled anti-human Siglec-9 and mouse IgG1- κ (BD Biosciences, San Jose, Calif., USA). The cultured cells were washed with Dulbecco's phosphate-buffered saline (PBS) solution containing 0.1% bovine serum albumin and then incubated with FITC-labeled anti-Siglec-9 or IgG1- κ antibodies at 4°C for 30 min. The labeled cells were analyzed with a Cytomics™ FC500 flow cytometer (Beckman Coulter, Hialeah, Fla., USA) using CXP software (Beckman Coulter).

RBC Binding Assay

Human blood was obtained from healthy volunteers. After centrifugation at 500 g, the RBCs were washed 3 times with PBS buffer. Binding assays were performed 2 days after the transfection of Siglec-9 and the mutants into the COS-7 cells. Then, 10 μ l

of compacted RBCs was resuspended with 490 μ l of 0.05 M HEPES buffer and added to the COS-7 cells, which had been pre-treated with 10 mU of neuraminidase (Sigma, St. Louis, Mo., USA) for 30 min at 37°C. After incubation, the unbound RBCs were removed with 3 PBS washes, and the bound RBCs were examined immediately under an inverted microscope (Leica, Wetzlar, Germany). Ten random fields were photographed for the 3 independent experiments. The number of RBC rosettes, which were defined as COS-7 cells binding more than 5 RBCs per field, was counted in 30 fields. The data were analyzed using an unpaired t test (SPSS 13.0 software) and considered statistically significant if $p < 0.05$.

Western Blot Analysis

Jurkat cells (2×10^6) were incubated either for 20 min with 5 μ g/ml of anti-CD3 antibody or for 10 min with 20 ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma). The cells were then homogenized in ice-cold homogenization buffer. Equal amounts (30 μ g) of the extracted protein were resolved with 8% SDS-PAGE and transferred to nitrocellulose membranes. After incubation in a blocking solution, the membranes were incubated overnight at 4°C with 1:1,000 diluted anti-phospho-ZAP-70 and anti-ZAP-70 antibodies (rabbit polyclonal; Cell Signaling Technology, Beverly, Mass., USA). The membranes were then further incubated with anti-rabbit horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) and treated with an enhanced chemiluminescence solution (Pierce kit; Rockford, Ill., USA). The signals were then captured on an Image Reader (LAS-3000; Fuji Photo Film, Tokyo,

Japan), and the protein bands were analyzed using Multi Gauge version 3.0 software (Fuji Photo Film).

IL-2 Expression Level Measurement

For the stimulation experiments, the Jurkat cells (1×10^6) were cultured with 20 ng/ml of PMA and 1 μ M of ionomycin (Calbiochem, La Jolla, Calif., USA) for the indicated period. Total RNA from the Jurkat cells, cultured under each condition, was obtained with Trizol Reagent (Invitrogen). Reverse transcription of the RNA was performed using a First Strand cDNA Synthesis Kit for RT-PCR (AMV; Boehringer Mannheim, Germany). The level of IL-2 mRNA relative to GAPDH was measured by quantitative real-time PCR using the LightCycler real-time PCR System (Roche). The primers used were as follows: IL-2: 5'-CACCAG-GATGCTCACATTTAAGTT-3' (forward) and 5'-GTGTCCTGA-GTTCTTCTTCTAGACACT-3' (reverse); GAPDH: 5'-TCCACT-GGCGTCTTACC-3' (forward) and 5'-GGCAGAGATGATGAC CTTT-3' (reverse).

To measure the amount of IL-2 at the protein level, the culture supernatants were collected and stored at -70°C . The level of IL-2 was measured using commercial ELISA kits (R&D Systems, Minneapolis, Minn., USA) according to the manufacturer's instructions.

Results

Involvement Only of the A391C (K131Q) Mutant in the Carbohydrate Binding

In human Siglec-9, 8 nonsynonymous SNPs have been reported without functional studies. In this study, we examined the position of these 8 SNPs by homology modeling. The plausible homology model structure of Siglec-9 is presented in figure 1a. The location of the SNPs was predicted for only 4 residues; K100E, S125N and K131Q could be located in the carbohydrate recognition domain and N147K could be positioned in the Ig-like domain. In

the case of the K131Q residue, it could directly interact with a carbohydrate molecule, whereas the K100E and S125N residues could not interact with carbohydrates, even if these residues were positioned in the carbohydrate recognition domain.

The docking model of Siglec-9 and the carbohydrate was generated (fig. 1b). The wild-type Lys¹³¹ side chain could form a hydrogen bond with a hydroxyl group of the carbohydrate molecule, whereas the Gln¹³¹ mutant could have a weak hydrogen bond interaction with a hydroxyl group of the carbohydrate molecule.

Reduced RBC Binding of K131Q-Transfected COS-7 Cells

To investigate their ability to bind with sialic acid in human RBCs, the COS-7 cells were transiently transfected with the full-length wild-type Siglec-9 or the 8 mutants K131Q, K100E, S125N, N147K, A315E, A316D, V349A, and Y45*. Before comparing the binding activity of the wild type and the mutants, the expression levels from the transient transfection were confirmed to be similar in the wild-type- and mutant-transfected cells (fig. 2a). In one mutant, Y45*, full-length Siglec-9 was not formed because the tyrosine residue was changed into a termination codon. The only mutant in which transfected cells showed significantly ($p = 0.002$) reduced binding with RBCs compared to the wild type was K131Q (fig. 2b, c).

Increased ZAP-70 Phosphorylation and IL-2 Production by K131Q

Siglec-9 has been shown to negatively regulate TCR signaling associated with ligand binding [16]. The engagement of TCR leads to the transcription of immune response genes, such as IL-2, through ZAP-70, a Syk kinase [21, 22]. Therefore, the effects of wild-type Siglec-9 and K131Q on TCR signaling were evaluated by the analysis of (1) phosphorylation of Tyr³¹⁹ with ZAP-70 and (2) the production of IL-2.

The expression levels of each gene were similar in comparisons between the Jurkat cells transiently transfected with the wild type and those with the mutant (fig. 3a). The induction of TCR engagement, with anti-CD3 (5 μ g/ml) or PMA (50 ng/ml) [23] in the transfected Jurkat cells, showed that the phosphorylation of ZAP-70 Tyr³¹⁹ increased in the mutant K131Q-expressing cells compared to the wild-type-expressing cells (fig. 3b, c).

Production of the immune response gene IL-2 was compared in the transfected cells with PMA stimulation by quantitative real-time PCR and ELISA. In the levels of mRNA expression, a markedly reduced inhibition was de-

Fig. 2. RBC binding assay. COS-7 cells were transiently transfected with the wild type or each mutant of Siglec-9, or a control vector (mock), as described in Materials and Methods. **a** Transfection efficiency was evaluated by flow cytometry. The levels of surface expression of the transfected cells, indicated by the filled histogram, were similar in the wild type and mutants. **b** In the RBC binding assay, the number of rosette-forming cells, i.e. those binding more than 5 RBCs per field, was counted by photographing 30 random fields. The representative photograph shows reduced numbers of rosette-forming cells, indicated by the arrows, in the K131Q mutant compared to the wild type. Scale bar = 50 μ m. **c** The data represent the mean $\pm$ SD of the number of RBC rosettes in the wild type and mutants. Significantly less binding was observed only in the K131Q mutant. * $p < 0.005$ was analyzed using an unpaired t test.

Fig. 3. ZAP-70 phosphorylation. Wild-type Siglec-9 and mutant K131Q genes were transiently transfected into Jurkat cells, with an empty vector (mock) as the control. **a** Transfection efficiency using flow cytometry was similar in the wild type and the mutant. **b** In both the Western blot analysis and a densitometry evaluation, a reduced inhibition of ZAP-70 phosphorylation was detected in the mutant-transfected cells compared to the wild-type-transfected cells, which were stimulated with 5 μ g/ml of anti-CD3 antibody for 20 min. The densitometry evaluation analyzed the ratios of ZAP-70 phosphorylation to total ZAP-70. The results were obtained from 3 independent experiments with each of the gene-transfected cells. The error bars represent the SD (n = 3). **c** Treatment with 20 ng of PMA for 10 min showed similar results by Western blot analysis and densitometry evaluation. The error bars represent the SD (n = 3). * $p < 0.05$ using an unpaired t test.

tected in the K131Q cells compared to the wild-type-transfected cells (fig. 4a). The levels of protein expression showed patterns similar to those of the mRNA expression (fig. 4b).

Discussion

The results of this study demonstrate the novel function of SNP on the ligand binding site of Siglec-9 through a molecular modeling analysis, RBC binding assay, and TCR signaling analysis. One of the 8 mutants, which had the amino acid lysine residue (Lys¹³¹) changed to a glutamine residue (Gln¹³¹) and was speculated to interact directly with a carbohydrate molecule resulted in reduced RBC binding, with increased ZAP-70 phosphorylation

and IL-2 production compared to the wild-type gene, indicating a reduced inhibition of immune reactions.

Analysis of the Siglec-9 homology model structure revealed that 4 amino acid residues of the 8 SNPs were in the binding sites. Among them, N147K was located in the Ig-like domain but not in the carbohydrate binding domain, and 2 SNPs, K100E and S125N, did not contribute to carbohydrate binding, although they were included in the carbohydrate recognition domain. In the mutant (dbSNP accession No. rs16988910) in which amino acid Lys(K)¹³¹ changes into Gln(Q)¹³¹, the length of the Lys(K)¹³¹ side chain seemed to be long enough to make a tight hydrogen bond with the galactose ring, whereas that of the Gln(Q)¹³¹ side chain did not, resulting in a reduction of the binding affinity to the ligand. Similar results

Fig. 4. IL-2 production. The same Jurkat cells transfected with each gene were stimulated with 20 ng of PMA and 1 μ M ionomycin for the indicated time. **a** The expression levels of mRNA, quantified by real-time PCR, showed reduced inhibition in the mutant compared to the wild-type-transfected cells with 4 h of stimulation. The data represent the mean $\pm$ SD from 3 indepen-

dent experiments. **b** The amount of protein in the culture supernatant was measured sequentially by ELISA at 2, 8, and 24 h. The extent of the inhibition was clearly reduced in the mutant compared to the wild type at every point in time measured. Data represent the mean $\pm$ SD from 3 independent experiments. * $p < 0.05$ was analyzed using an unpaired t test.

could be inferred from the study results of Siglec-7; the structure of this CD33-related Siglec has been determined [6] and it represents a good candidate for structure-based inhibitor design in site-directed mutagenesis and binding assays. The mutagenesis and binding assays of Siglec-7 have shown that residues Asn¹³³ and Lys¹³⁵ may play a genuine role in ligand binding [24]. The mutation of residues including Lys¹³⁵ into alanine has been shown to lose the ability to bind [25]. The mutation of Lys¹³⁵ of Siglec-7 is applicable to Lys¹³¹ of Siglec-9 in terms of the alignment [26], although the A391C polymorphism changed amino acid residue Gln¹³¹.

RBC binding was significantly ($p = 0.002$) inhibited in the only mutant Gln¹³¹ transfected into the COS-7 cells, as was the expected result in the molecular modeling analysis (fig. 2). The result showing that an active ligand domain is required whenever Siglec-9 negatively regulates TCR signaling at multiple steps in the signaling pathway [16] may suggest that the K131Q mutant could reduce the inhibition of TCR activation. Jurkat cells are known to be ideal for the analysis of the effects of Siglec-9 on TCR signaling because there is no Siglec-9 expression on the cells. However, TCR signaling may not be significant for the analysis of Siglec-9 effects because Siglec-9 is not expressed by most T cells. Nonetheless, this study focused on the binding activity of the mutants to sialic acid. Moreover, the sialic-acid-binding activity regulated by Siglec-9 has been examined only through TCR signaling in Jurkat cells [16], which is why Jurkat cells were used for this study. TCR activation recruits and activates ZAP-70, a Syk kinase. TCR is com-

posed of TCR and CD3 dimers, and TCR engagement has induced only anti-CD3 [16]. Treatment with PMA, a direct activator of protein kinase C, also triggers TCR through the activation of Ras in T cells [27]. Moreover, treatment with either the anti-CD3 antibody (fig. 3b) or PMA plus calcium ionophore (fig. 3c) activated ZAP-70 in this study. Since Siglec-9 after ligand binding downregulates TCR activation by the recruitment of SHP-1 [16], the reduced binding would be expected to less inhibit ZAP-70 phosphorylation at Tyr³¹⁹ [16]. In fact, the inhibition levels of ZAP-70 phosphorylation in the mutant K131Q-transfected Jurkat cells were significantly lower than those of the wild-type gene-transfected cells, regardless of the treatment method (anti-CD3 or PMA plus ionophore) (fig. 3b, c).

The end product of ZAP-70 phosphorylation is the NFAT-mediated transcription of immune response genes, such as IL-2 or IFN- γ [8]. However, treatment with the anti-CD3 antibody alone was not enough to measure IL-2 (data not shown). Although CD28 costimulation plays an essential role in IL-2 production in primary T cell activation [28, 29], it was not investigated in this study. Instead of CD28 stimulation, treatment with PMA plus calcium ionophore was tried since protein kinase C-dependent activation of the Ras signal transduction cascade has been reported to be essential for the induction of the IL-2 promoter during the stimulation of T cells via the TCR [30]. In fact, the production of IL-2 was confirmed on both mRNA (fig. 4a) and protein (fig. 4b) levels. Moreover, the K131Q mutant inhibited IL-2 production less compared to the wild type (fig. 4).

Overall, this study showed that mutant A391C (K131Q) of Siglec-9 reduced sialic-acid-ligand-binding as well as the inhibition of TCR-mediated signaling represented by ZAP-70 phosphorylation and IL-2 production compared to the wild type.

Acknowledgement

This study was supported by a grant (03-PJ10-PG13-GD01-0002) from the Korea Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea.

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