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Assessment of sialic acid diversity in cancer- and non-cancer related CA125 antigen using sialic acid-binding Ig-like lectins (Siglecs)

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Abstract. This study was aimed at obtaining insight into the diversity of sialic acids in cancer- and non-cancer-related CA125 antigen, tumour marker of serous ovarian cancer. Starting from available data suggesting the possible relevance of sialic acids for discriminating CA125 antigens of different origin, we have employed a new experimental approach based on the use of human sialic acid-binding Ig-like lectins, Siglecs, as tools for the investigation of sialylation.
Siglec–2, belonging to the group of evolutionarily conserved Siglecs, and Siglec–3, −6, −7, −9 and −10, which are CD33-like Siglecs, were probed in solid-phase binding assays with cancer-related CA125 antigens from pleural fluid of patients with ovarian carcinoma (pfCA125), the OVCAR-3 ovarian carcinoma cell line (clCA125) and a non-cancer-related CA125 antigen, i.e. pregnancy-associated pCA125 antigen.
All Siglecs used showed detectable binding to pCA125 antigen. Siglec–3, Siglec–7 and Siglec–2 exhibited moderately stronger binding to pCA125 antigen than the others. In contrast to this, Siglec–2 and Siglec–3 preferentially recognized pfCA125 with greater total binding than for pCA125, whereas Siglec–9 and Siglec–10 were highly selective for clCA125.
Siglecs promise to be powerful tools for discriminating CA125 of different origin and could propagate further research on other molecular markers of biomedical and diagnostic importance.

Keywords: CA125 antigen, glycans, sialic acid, Siglec

1. Introduction

Sialic acids (Sia) are important structural determinants influencing the physical and functional properties of molecules either by blocking existing functions or by introducing completely new ones [22]. They occur in various modifications and combinations of different linkages and are mostly located terminally on N- and O-glycans, but can also occupy internal positions within glycans, as polysialic acids [3,13]. Generally, Sia can act as ligands for many biologically active substances, but can also be anti-recognition agents of distinct antigenic determinants [22]. Changes in sialylation occur during normal physiological processes, such as growth and development, but also in numerous pathophysiological conditions [1,19]. Neoplastic transformation is accompanied with pronounced changes in sialylation and some sialylated glycotopes are known as tumor markers for cancer detection or as prognostic markers for predicting metastatic potential [21]. Cancer-associated structural alterations regarding the type and composition of Sia are found on different glycoconjugates, including mucins [10]. This study was aimed at obtaining insight into the diversity of sialic acids in cancer- and non-cancer-related CA125 antigen, which is defined as the extracellular part of MUC16, and is a tumour marker of serous ovarian cancer.

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CA125 antigen is composed of tandem repeats between N- and C-terminal regions and glycans comprise about 28% of its molecular mass [6,18,24–26]. The N-terminal region is heavily decorated with O-linked oligosaccharide chains and, untypical of mucins in general, complex and high-mannose type N-linked sugars are also present. Mass spectrometry analysis of glycosylation of CA125 antigen expressed in OVCAR-3 cell culture has demonstrated that sialylation mostly concerns O-glycans with mono- and disialylated type 1 and type 2 cores, while sialylation of N-glycans is relatively low [18]. Employing the lectin-based approach, comparative analysis of CA125 antigen from the OVCAR-3 cell line and pregnancy-associated CA125 antigens from human amniotic fluid or placental tissue have shown the presence of both Neu5Acα2,3 and Neu5Acα2,6, the latter increasing in the cancer-related antigen [14,15]. The existence of differently sialylated subpopulations of CA125 antigen, deduced from corresponding elution patterns, also suggested the presence of mono- and di-sialylated structures and that Sia may be linked to different underlying glycans.

Starting from the available data suggesting the possible relevance of Sia for discriminating CA125 antigen of different origin, we have employed a new experimental approach based on the use of human sialic acid-binding Ig-like lectins, Siglecs, as tools for investigating Sia diversity. Siglecs have not been used for this purpose so far, but their distinct carbohydrate specificities and human origin possibly allow a higher potential in discriminating different CA125 glycoforms than the widely used Sia-binding plant lectins. Siglecs form the major subfamily of I-type lectins, comprising proteins other than antibodies and T-cell receptors that mediate glycan recognition via immunoglobulin (Ig)-like domains [2]. They include several proteins divided into an evolutionarily conserved subgroup containing Siglec–1, -2 and -4, and the CD33/Siglec–3 related subgroup, where Siglec–3 and Siglec–5 – Siglec–13 belong [2]. Siglecs are supposed to recognize different linkages and presentations of Sia molecules, but whereas there are defined structures bound by evolutionarily conserved forms, CD33-like Siglecs often bind more than one aspect of Sia [7,20]. Thus, Siglec–2 of the evolutionarily conserved group and Siglec–3, -6, -7, -9 and -10 of the CD33-like group were probed in solid-phase binding assay with cancer-related CA125 antigens from pleural fluid of patients with ovarian carcinoma or OVCAR-3 ovarian carcinoma cell line and non-cancer related CA125 antigen, i.e. pregnancy-associated CA125 antigen. The results obtained indicate distinct Siglec–binding patterns of CA125 antigens which were more selective than the pattern obtained with plant sialic acid-binding lectins.

2. Materials and methods

2.1. Materials

CA125 isolated from the pleural fluid of patients with ovarian carcinoma (pfCA125) and CA125 isolated from OVCAR-3 ovarian carcinoma cell line (clCA125) were purchased from Meridian Life Science (Saco, Maryland, USA). The concentration of pfCA125 antigen was 73,778 IU/mL, 2.6 OD_{280} Units, and it contained $<$ 1% CA19-9 and 1% CA15-3. The concentration of clCA125 antigen was 150,522 IU/mL, 0.469 OD_{280} Units, and it contained 0.2% CA19-9 and $<$ 1% CA15-3. Pregnancy-associated CA125 antigen (pCA125) was isolated from first trimester human placental extract [14]. The concentration of pCA125 antigen was 5,000 IU/mL (CIS Biointernational, ELSA CA125 II assay), 0.406 OD_{280} Units, and it contained $<$ 0.2% CA19-9 (CIS Biointernational, ELSA CA19-9) and $<$ 0.01% CA15-3 (MUC1) (CIS Biointernational, ELSA CA15-3).

Recombinant human Siglec/Fc chimeras (-2, -3, -6, -7, -9 and -10) were purchased from R&D systems (Minneapolis, Minnesota, USA). Biotinylated plant lectins SNA (*Sambucus nigra* agglutinin) and MAA II (*Maackia amurensis* agglutinin II), Elite Vectastain ABC kit and DAB substrate kit were from Vector laboratories (Burlinghame, USA). Protein A-HRPO conjugate was from INEP (Belgrade, Serbia). Microtiter polystyrene plates with 96 wells (COSTAR) were purchased from Corning International (New York, USA). 3,3',5,5'- tetramethylbenzidine (TMB) was from HUMAN GmbH (Wiesbaden, Germany). Roti black P kit was from Carl Roth GambH+Co. Karlsruhe, Germany). Molecular mass standards were purchased from Bio-Rad (Hercules, USA).

All other chemicals were reagent grade.

2.2. Methods

2.2.1. SDS-PAGE

CA125 antigens were resolved by SDS-PAGE, according to Laemmli [27], on 4% stacking gel and 10% separating gel. Gel was stained with silver nitrate using a Roti black P kit, according to the manufacturers instructions.

2.2.2. Binding of sialic-acid specific plant lectins to immobilized CA125 antigens

In the lectin-binding assay, serial dilutions (50 μL/well) of corresponding CA125 antigens, in 0.05 M carbonate buffer, pH 9.5, concentration range 7–500 IU/mL, were immobilized onto a polystyrene plate overnight at +4°C. Excess protein was rinsed away with 0.05 M PBS, pH 7.2. Plates were blocked with 0.5% BSA and again rinsed. Biotinylated SNA or MAA II (50 μL/well, at 2.5 μg/mL constant concentration) was added. After incubation for 2 hours at room temperature, plates were rinsed and Vectastain Elite ABC reagent added. After 30 min incubation and another washing step, TMB substrate solution was added and incubated for 10 min. The reaction was stopped with 0.16 M H_2SO_4. The absorbance was measured at 450 nm on a Wallac 1420 Multilabel Counter PerkinElmer, Monza, Italy. Binding of SNA and MAA II to control wells without CA125 antigens was considered as non-specific binding (NSB). Binding of SNA and MAA II to immobilized CA125 antigens was expressed relative to NSB.

In parallel, CA125 antigens subjected to mild periodate treatment (at 500 IU/mL) were analyzed. Thus, prior to blocking, immobilized CA125 antigens were oxidized with 2 mM $NaIO_4$ (150 μL/well) for 30 min. The reaction was stopped with 8% glycerol (20 μL/well). After a washing step with 0.05 M PBS, pH 7.2, antigens were incubated with 20 mM of $NaBH_4$ (150 μL/well) for 30 min. Plant lectins were bound to periodate-treated immobilized CA125 antigens as described above and expressed as a percentage of the binding to untreated CA125 antigens.

2.2.3. Binding of human Siglecs to immobilized CA125 antigens

The binding assay for Siglec/Fc chimera was performed with slight modifications as previously described [17]. Serial dilutions (50 μL/well) of corresponding CA125 antigens, in 0.05 M carbonate buffer, pH 9.5 (concentration range 7–500 IU/mL), were immobilized onto a polystyrene plate overnight at +4°C. Excess protein was removed by rinsing with 0.05 M PBS, pH 7.2. Plates were blocked with 0.5% BSA and again rinsed. Siglec/Fc chimera (50 μL/well, at 2.5 μg/mL constant concentration) was added and incubated for 2 hours at room temperature. The unbound protein was rinsed away as previously, and protein A-HRPO conjugate (50 μL/well, at 0.3 μg/mL constant concentration) was added. After incubating for 2 hours at room temperature and a washing step, TMB substrate solution was added and the plates incubated for 10 min. The reaction was stopped with 0.16 M H_2SO_4 and the absorbance measured at 450 nm on a Wallac 1420 Multilabel Counter PerkinElmer, Monza, Italy. Binding of Siglec/Fc chimeras to control wells (without CA125 antigens) was considered as NSB. Binding of Siglec/Fc chimeras to immobilized CA125 antigens was expressed relative to NSB.

In parallel, periodate-treated CA125 antigens were assayed as described for native, non-treated antigens.

3. Results

CA125 antigens of different origin (Fig. 1.) were analyzed in a lectin-binding assay. The concentration-dependent binding patterns of SNA and MAA II to immobilized CA125 antigens are shown in Fig. 2 (*A1–C1*). The binding curves for pCA125 (Fig. 2, A1) and pfCA125 (Fig. 2, B1) were similar over the entire concentration range tested, with no difference in respect to the lectins used, but with higher maximal binding in the latter. In contrast to this, saturation was reached at much lower concentrations of clCA125 for both SNA and MAA II (Fig. 2, C1). Moreover, the maximal binding of MAA II was distinctly higher than that of SNA, but without exceeding that observed for pfCA125. The interaction of SNA with all examined antigens was sensitive to mild periodate treatment, which reduced binding by 60–70%, whereas MAA II was affected to a much lesser extent (Fig. 2 (A2–C2)).

Binding of recombinant human Siglec/Fc chimeras to the different CA125 antigens showed distinct patterns Fig. 3 (A1–C1). All Siglecs tested exhibited moderate binding to pCA125, but at higher antigen concentrations (125–500 IU/mL), Siglec–3 and −7 and, to a lesser extent Siglec–2, were more reactive (Fig. 3, A1). Periodate treatment of pCA125 reduced the level of binding of Siglec–2, −3 and −7 by 14%, 7% and 5%, respectively (Fig. 3, A2).

As for pfCA125, Siglec–2 and −3, and to a lesser extent Siglec–6, showed the highest binding (Fig. 3, B1), while mild periodate treatment reduced it by 18%, 20% and 3%, respectively (Fig. 3, B2).

Siglec–9 and Siglec–10 were highly selective to clCA125 antigen (Fig. 3, C1). In comparison to interactions of the selected Siglecs with the other CA125 antigens, periodate treatment had considerable effects on Siglec–9 and Siglec–10 binding to clCA125, reducing it by 40% and 56%, respectively (Fig. 3, C2).

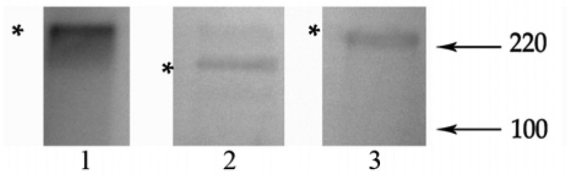

Fig. 1. Molecular forms of cancer- and non-cancer related CA125 antigens. Antigens (5000 IU/mL) were separated by SDS-PAGE on 4% stacking gel and 10% separating gel and stained with silver. Molecular masses of markers are indicated. (1) pCA125 – pregnancy-associated CA125 antigen; (2) pfCA125 – CA125 antigen from pleural fluid of ovarian cancer patients; (3) clCA125 – CA125 antigen from OVCAR-3 cell line.

4. Discussion

The data given here demonstrate the diversity of sialic acid presentations in cancer-related and non-cancer-related CA125 antigens, through which they are specifically recognized by sialic-acid-binding lectins of endogenous and exogenous origin. Diversity generated by different α-linkages was found in both groups of CA125 antigens studied, but the binding profiles of SNA, (recognizing Neu5Acα2,6Gal/GalNAc) and MAA II (recognizing Neu5Acα2,3Galβ1,4GlcNAc/Glc) were similar for all tested antigens. However, more selective and discriminative binding patterns were obtained with human Siglecs, a distinct group of sialic acid-binding lectins. They support the existence of different glycosidic linkages with various substitutions present in specific spacing combinations in the oligosaccharide chains of each CA125 antigen.

Thus, under our experimental conditions, all Siglecs used showed detectable binding to pCA125 antigen. Siglec–3, Siglec–7 and Siglec–2 exhibited moderately stronger binding to pCA125 antigen than to the others. In contrast to this, Siglec–2 and Siglec–3 preferentially recognized pfCA125 with greater total binding than for pCA125, whereas Siglec–9 and Siglec–10 were highly selective for clCA125. All these interactions were more or less sensitive to mild periodate treatment, which truncates the glycerol-like side chain of Sias. Since, the binding site of Siglecs interacts with glycerol side chain of Sia, oxidative cleavage of the glycerol side chain is thought to provides most stringent controls for Sia-dependent binding of Siglecs [17].

The Siglecs used here were recombinant chimera proteins composed of the carbohydrate-binding domain fused to the Fc region of human IgG. This increases their affinity in comparison to native molecules due to polymerization i.e. a clustering effect. The available literature underlines the high selectivity of Siglec–2, which recognises only Neu5Acα2,6Gal [2,4,12] and it is usually compared to SNA. Periodate treatment abolished the binding of both lectins, but in spite of this, the importance of Sia presentation for high affinity interactions was demonstrated by the finding that they differentially agglutinate erythrocytes of different blood types [8]. Moreover, SNA can bind sialic acid with or without 9-O-acetyl groups, whereas Siglec–2 is blocked by 9-O-acetylation [8,9]. The glycoprotein, fetuin, was reported to inhibit Siglec–2 [17], which was found to bind both pCA125 and pfCA125. Interestingly enough, we have recently demonstrated a placental carbohydrate-binding IgG as an endogenous ligand for pregnancy-associated CA125 antigen [5]. This IgG could be specifically enriched on a fetuin-Sepharose column and it might recognize the sialoglycotopes of CA125 antigen corresponding to those recognized by Siglec–2.

Siglec–7, which bound pCA125, also, prefers α2,6 linked Sias. Neu5Acα2,6Galβ1,4Glc showing the strongest binding to Siglec–7, is similar to Siaα2,6Galβ1,4GlcNAc, which is a ligand for Siglec–2 [23]. As for Siglec–3, it binds Neu5Acα2,6Gal with higher affinity than Neu5Acα2,3Gal, but can also recognize sialyl-Tn antigen and may, in a limited physiological context, slightly be affected by 9-O-acetylation [7,20]. The structurally related Siglec–6, also, interacts with Neu5Acα2,6Gal and it is very selective in recognizing sialyl-Tn antigen, with key factors being density, grouping and optimal resolution of the antigen [7]. In contrast to the other Siglecs, it is known that periodate treatment does not alter binding of Siglec–6 [7,8], as observed when probed with pfCA125 antigen.

Thus, although the results obtained suggested different residues involved in Sia recognition in pCA125 and pfCA125, they exhibited similarity in the type of Siglec selecting them. However, clCA125 was clearly different as a ligand. Only Siglec–9 and Siglec–10 recognized clCA125 antigen. Siglec–9 and Siglec–10 have similar binding specificity, recognizing both α2,3 and α2,6 linked Sia, but with slight preference towards Neu5Acα2,3Galβ1,4GlcNAc, which is present in N-glycans [20]. The most striking difference is the

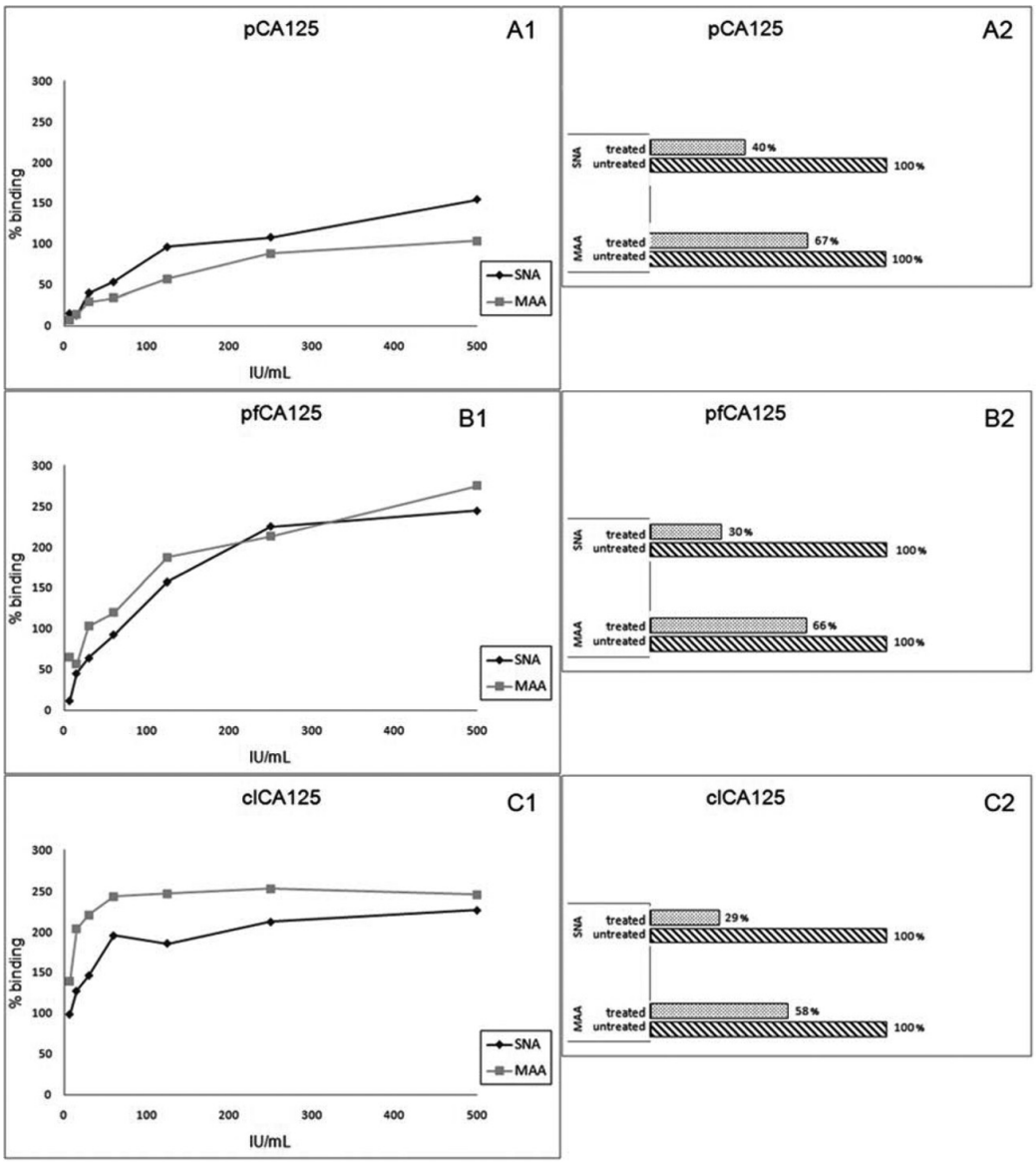

Fig. 2. Binding of SNA and MAA II to cancer- and non-cancer related CA125 antigens. Immobilized CA125 antigens (7–500 IU/mL) were allowed to react with biotinylated SNA or MAA II for 2 hours at room temperature. The unbound material was washed out followed by addition of Vectastain Elite ABC reagent and a TMB substrate solution. The presented results are mean values of two independent experiments. Standard error ranged from 5–17% (data not shown). Panels A1, B1, C1: Native CA125 antigens; binding of SNA and MAA II was expressed as a percentage, relative to non-specific binding. Panels A2, B2, C2: Periodate-treated CA125 antigens (500 IU/mL); binding of SNA and MAA II was expressed as a percentage of the binding to native CA125 antigens. pCA125 – pregnancy-associated CA125 antigen (A), pfCA125 – CA125 antigen from pleural fluid of ovarian cancer patients (B), clCA125 – CA125 antigen from OVCAR-3 cell line (C).

influence of fucosylation. Thus, introduction of fucose and the formation of sialyl-Le^x completely abolish Siglec–10 binding, but do not influence Siglec–9 binding. MAA II reactivity clearly demonstrated the presence of Neu5Acα2,3Gal in all tested CA125 antigens. It is known that periodate treatment does not reduce MAA II binding [8], whereas Siglec–9 and Siglec–10 are sensitive. Comparable effects were observed when clCA125 antigen was probed in our experimental system.

In contrast to pCA125 and pfCA125, clCA125 was subjected to detailed glycosylation analysis using chemical methods which degrade the molecule. This indicated that the level of sialylation in N-glycans is

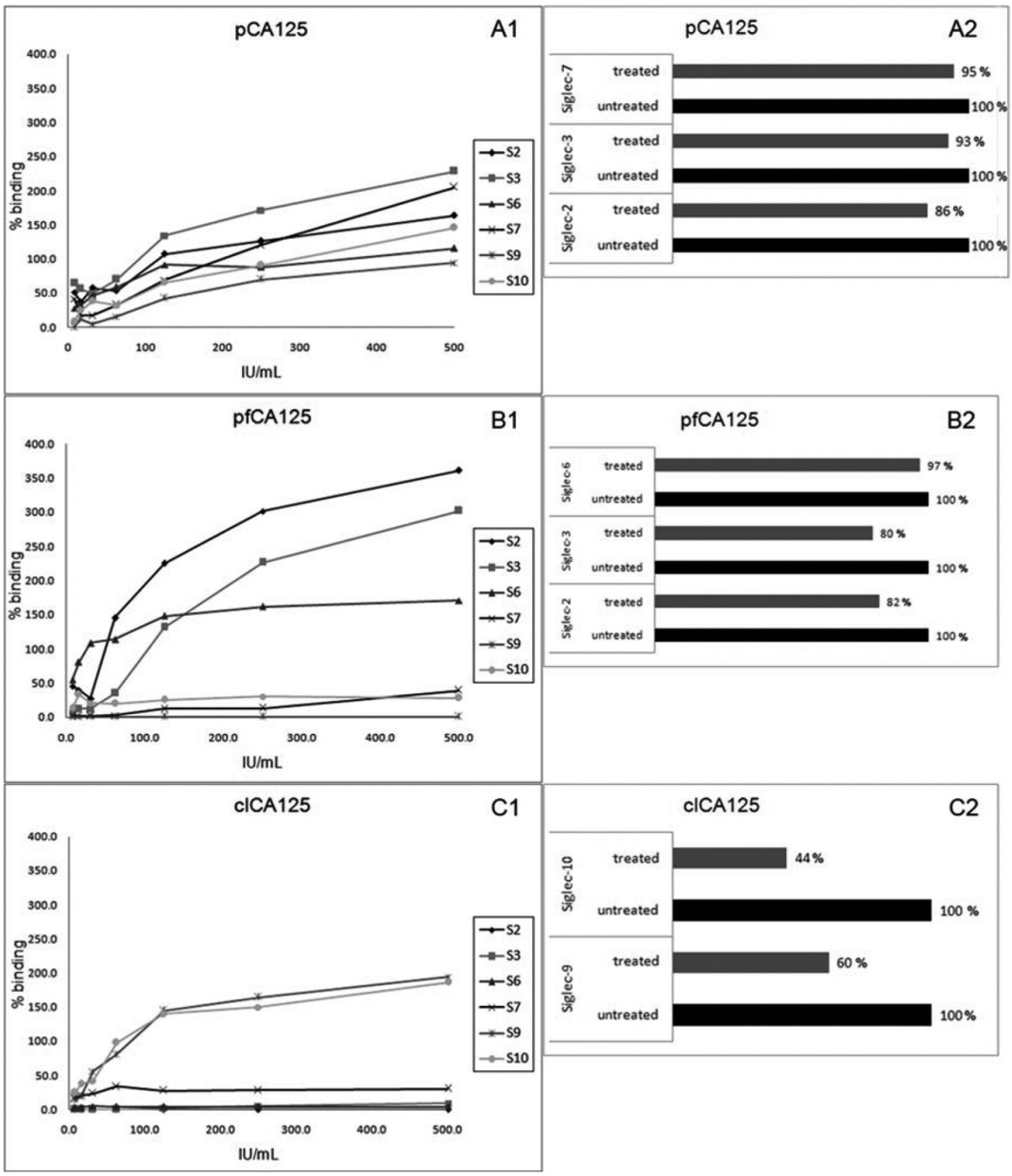

Fig. 3. Binding of human Siglecs to cancer- and non-cancer related CA125 antigens. Immobilized CA125 antigens (7–500 IU/mL) were allowed to react with corresponding Siglec/Fc chimeras for 2 hours at room temperature. The unbound material was washed out followed by addition of Protein A-HRPO and a TMB substrate solution. The presented results are mean values of two independent experiments. Standard error ranged from 4–13% (data not shown). Panels A1, B1, C1: Native CA125 antigens; binding of human Siglecs/Fc chimeras was expressed as a percentage, relative to non-specific binding. Panels A2, B2, C2: Periodate-treated CA125 antigens (500 IU/mL). Binding of selected human Siglecs/Fc chimeras was expressed as a percentage of the binding to native CA125 antigens. pCA125 – pregnancy-associated CA125 antigen (A), pfCA125 – CA125 antigen from pleural fluid of ovarian cancer patients (B), clCA125 – CA125 antigen from OVCAR-3 cell line (C).

low, and no component carries more than one Sia [18], which can be correlated with the obtained preference of Siglec–9 and −10 for clCA125 antigen since monosialylated N-glycans are preferred ligands for these Siglecs [20]. As for Le^x, the presence of which was unequivocally demonstrated on clCA125 [18], no clear conclusion can be drawn about its sialylated form i.e. sialyl-Le^x, based on the obtained binding pattern of Siglecs.

Generally, when considering reactivities of Siglecs

and relating them to specific carbohydrate structures, it should be born in mind that absence of binding does not necessarily imply absence of the cognate glycan structure in the examined molecule. Interaction between CA125 antigen and Siglecs has not been studied. However, the results of a study on an NK cell subset indicated that CA125 antigen in cancer patients is bound to these cells via Siglec–9 [11,16] which agrees with our findings for clCA125.

The approach used in this study was based on recognition of native carbohydrate structures containing sialic acid. In the context of structural integrity, the accessibility of such residues is relevant for *in vivo* interactions. In addition, they may also be valuable as markers for targeting in different detection systems and for clinical purposes of differentiating between various malignant and benign conditions associated with elevated CA125 concentrations in sera or other body fluids. Thus, although this is beyond the scope of this investigation, the potential of CA125 antigen to interact with certain Siglecs can be put in the context of discrete biological functions of each Siglec. Further research is needed to determine whether these interactions actually occur *in vivo*. Regardless of what their natural ligands are, Siglecs promise to be powerful tools for discriminating CA125 antigens of different origin and could propagate further research on other molecular markers of biomedical and diagnostic importance.

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