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Interaction of chondroitin sulfate and dermatan sulfate from various biological sources with heparin-binding growth factors and cytokines

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Abstract Chondroitin sulfate (CS) and dermatan sulfate (DS) interact with various extracellular molecules such as growth factors, cytokines/chemokines, neurotrophic factors, morphogens, and viral proteins, thereby playing roles in a variety of biological processes including cell adhesion, proliferation, tissue morphogenesis, neurite outgrowth, infections, and inflammation/leukocyte trafficking. CS/DS are modified with sulfate groups at C-2 of uronic acid residues as well as C-4 and/or C-6 of *N*-acetyl-D-galactosamine residues, yielding enormous structural diversity, which enables the binding with numerous proteins. We have demonstrated that highly sulfated CS-E from squid cartilage, for example, interacts with heparin-binding proteins including midkine, pleiotrophin, and fibroblast growth factors expressed in brain with high affinity (*Kd* values in the nM range). Here, we analyzed the binding of CS and DS, which have a relatively low degree of sulfation and have been widely used as a nutraceutical and a drug for osteoarthritis etc., with a number of heparin-binding neurotrophic factors/cytokines using surface plasmon resonance (SPR) and structurally characterized the CS/DS chains. SPR showed that relatively low sulfated CS-A, DS, and CS-C also bound with significant affinity to midkine, pleiotrophin, hepatocyte growth factor, monokine-induced by interferon-γ, and stromal cell derived factor-1β, although the binding was less intense than that with highly sulfated CS-D and CS-E. These findings suggest that even low sulfated CS and/or DS chains may contain binding domains, which include fine sugar sequences with specific sulfation patterns, and that sugar sequences, conformations and electrostatic potential are more important than the simple degree of sulfation represented by disaccharide composition.

Keywords Chondroitin sulfate · Dermatan sulfate · Growth factors · Cytokines · Interaction analysis · Surface plasmon resonance

Abbreviations

CS	Chondroitin sulfate
DS	Dermatan sulfate
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
HIV	Human immunodeficiency virus
HGF	Hepatocyte growth factor
MIG	Monokine-induced by interferon-γ
MK	Midkine
PG	Proteoglycan
PTN	Pleiotrophin
RANTES	Regulated upon activation normal T cell express sequence
SDB-1β	Stromal cell derived factor-1β
SPR	Surface plasmon resonance

Introduction

Glycosaminoglycan (GAG) chains such as chondroitin sulfate (CS) and dermatan sulfate (DS) are covalently attached to specific core proteins, forming proteoglycans (PGs) [1, 2]. CS- and DS-PGs are ubiquitously distributed at cell surfaces and in extracellular matrices, and play roles through

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the GAG side chains in many biological processes such as cell proliferation, tissue morphogenesis, neurite outgrowth, infections of viruses/bacteria, inflammation/leukocyte trafficking, and the regulation of functions of various growth factors/cytokines [3–5].

Such functions are based on the enormous structural diversity of GAG chains. The sugar back bones of CS and DS are linear polysaccharides composed of the repeating disaccharide units, [-4GlcUAβ1-3GalNAcβ1-]n and [-4IdoUAα1-3GalNAcβ1-]n, where GlcUA, GalNAc, and IdoUA represent D-glucuronic acid, *N*-acetyl-D-galactosamine, and L-iduronic acid, respectively [1, 4]. Both CS and DS are frequently found as CS/DS hybrid chains in mammalian tissues. CS/DS chains are modified by specific sulfotransferases with a sulfate group at C-2 of the uronic acid residues as well as C-4 and/or C-6 of the GalNAc residues, yielding various kinds of monosulfated disaccharide units such as GlcUA/IdoUA-GalNAc(4-*O*-sulfate) or GlcUA/IdoUA-GalNAc(6-*O*-sulfate), which are abbreviated as A/iA- and C/iC-units, respectively. In addition, several disulfated units including GlcUA(2-*O*-sulfate)/IdoUA(2-*O*-sulfate)-GalNAc(6-*O*-sulfate) (D/iD-unit), GlcUA/IdoUA-GalNAc(4-*O*-, 6-*O*-sulfate) (E/iE-unit), and GlcUA(2-*O*-sulfate)/IdoUA(2-*O*-sulfate)-GalNAc(4-*O*-sulfate) (B/iB-unit) also exist [1, 4]. Combinations of these units can form a large number of complex sequences, which far outnumber nucleotides or peptides.

Distinct sulfation patterns are the structural basis for their biological functions and activities. For example, CS structures containing C-, D-, and E-units play roles in bone development, neuritogenesis, inflammation/leukocyte trafficking, viral infections, and tumor metastasis [5–10]. A DS structure containing iA-units on the decorin core protein modulates the formation of collagen bundles in skin [11]. Thus, to better understand the functions of CS/DS chains *in vivo*, an investigation of the structure-activity correlation of CS/DS chains interacting with functional proteins is desired. Highly sulfated CS-D and CS-E strongly interact with various growth or neurotrophic factors such as fibroblast growth factor (FGF)-2, FGF-10, FGF-18, midkine (MK), and pleiotrophin (PTN) [12, 13], as well as proinflamatory cytokines and chemokines like stromal cell derived factor-1β (SDF-1β) [5].

CS preparations from shark and porcine cartilage, for example, which are relatively low sulfated on average, have been widely utilized as a nutraceutical and a drug for osteoarthritis [14, 15]. However, the bioactivities of such low sulfated CS preparations have not been rigorously investigated. In this study, we investigated interactions of relatively low sulfated CS preparations from various biological sources with representative functional proteins including chemokines/cytokines. Chemokines are a family of cytokines or peptides that function as a chemoattractant by binding to members of the large family of G-protein-coupled receptors [16]. Chemokines, which are primarily basic proteins, bind to GAGs including CS, DS, and heparan sulfate (HS) [16], which are highly negatively charged polymers. However, the molecular basis of the interactions between chemokines and GAGs (*i.e.*, sulfation pattern; content of IdoUA; chain length etc.) remains to be investigated. In this study, surface plasmon resonance (SPR) was used to examine the effects of low sulfated CS and DS chains in addition to highly sulfated CS chains for comparison on the ability to mediate interactions with various growth factors and cytokines.

Materials and methods

Materials

The following sugars and enzymes were purchased from Seikagaku Corp. (Tokyo, Japan): various CS preparations including CS-A from whale cartilage, CS-B (DS) from porcine skin, CS-C from shark cartilage, CS-D from shark fin cartilage, and CS-E from squid cartilage; six unsaturated standard disaccharides derived from CS; chondroitinase ABC (EC 4.2.2.20) from *Proteus vulgaris*; chondroitinase AC-I (EC 4.2.2.5) from *Flavobacterium heparinum*; and chondroitinase AC-II (EC 4.2.2.5) from *Arthrobacter aurescens*. Chondroitinase B (EC 4.2.2.19) from *F. heparinum* was obtained from IBEX Technologies (Montreal, Canada). CS from porcine cartilage (CS-P) was provided by Zeria Pharmaceutical Co., Ltd. (Tokyo, Japan). A minute amount (1.3 %) of HS, which was contained in a commercial CS-B preparation from Seikagaku Corp. (1.3 %), was removed by digestion with a mixture of heparinases-I and –III, followed by anion-exchange HPLC [17]. Alternatively, the preparation was treated with nitrous acid at room temperature and at low pH (~1.5) as reported previously [18] for removal of HS chains.

Recombinant human PTN and hepatocyte growth factor (HGF) expressed in Sf21 insect cells were from R&D Systems (Minneapolis, MN). Recombinant human PTN, MK, Regulated upon activation normal T cell express sequence (RANTES), SDF-1β, macrophage inflammatory protein (MIP)-1α, and MIP-1β expressed in *E. coli* were from Wako Pure Chemical Industries (Osaka, Japan). Recombinant human monokine-induced by interferon gamma (MIG) expressed in *E.coli* was from Peprotech (London, UK). RANTES is generated by immune cells, and transported to sites of inflammation and infection, exhibiting in antimicrobial activity [19]. Furthermore, recent studies showed that RANTES is involved in the pathogenesis of HIV as well as West Nile virus [19]. On the other hand, SDF-1β binds to a receptor and is involved in a variety of processes such as

embryonic development, hematopoiesis, angiogenesis, cancer metastasis, HIV infection, and coronary artery disease (also atherosclerotic heart disease) [20]. MIG is involved in various immunoinflammatory diseases as well as neuroimmune diseases of the central nervous system [21].

Determination of the disaccharide composition of CS/DS chains

To determine the disaccharide composition, the commercial CS/DS preparations were digested with chondroitinase ABC, a mixture of chondroitinases AC-I and AC-II, or chondroitinase B, and each digest was labeled with a fluorophore, 2-aminobenzamide (2AB) as described previously [22]. The resultant 2AB derivatives of the unsaturated disaccharides were identified and quantified by anion-exchange HPLC on an amine-bound silica column as reported previously [17, 22].

Surface plasmon resonance analysis

The interaction of growth factors or cytokines with various types of CS and DS was examined using a BIAcore 2000 or T200 system (BIAcore AB, Uppsala, Sweden) as reported [23]. Briefly, CS and DS chains were biotinylated using biotin-LC-hydrazide and 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (Pierce) at their carboxy groups of the GlcUA residues in 100 mM MES-NaOH, pH 5.5 [12]. The labeling reaction was carried out overnight at room temperature. Each reaction mixture was applied to centrifugal filter devices, Amicon® Ultra-0.5 with a molecular mass cut-off of 3,000 Da (Millipore), to remove excess reagents. Hexuronic acid in each preparation was quantified by the carbazole method [24]. Biotinylated CS or DS chains (~1.0 ng) were individually immobilized on the surface of a streptavidin-derivatized sensor chip. The binding reactions were carried out at 25 °C, and the growth factors/cytokines, which were dissolved in the running buffer, 10 mM HEPES, 0.15 M NaCl, 3 mM EDTA, and 0.005 % (w/v) Tween 20, pH 7.4, were individually injected onto the sensor chips. Kinetic parameters were evaluated with BIAevalution software 4.1 (BIAcore AB) using a 1:1 binding model with mass transfer, which can also be applied for the "1:n" bindings such as the interaction of GAG with functional proteins provided the multiple binding sites have similar structures and affinity, and association and dissociation rate constants (k_a and k_d) as well as dissociation equilibrium constants (K_D) were determined. In addition, a heterogeneous binding model was used since the CS preparations used may contain two kinds of binding sites with different affinities.

Results

Disaccharide composition of CS and DS preparations

The disaccharide composition of CS/DS variants was determined by digestion with chondroitinases differing in specificity, followed by anion-exchange HPLC. The disaccharide composition of CS-A, CS-B, CS-C, CS-D, and CS-E preparations was consistent with previous observations (data not shown) [17]. The CS preparation from porcine cartilage (CS-P) was digested with chondroitinases differing in specificity, chondroitinase ABC, a mixture of chondroitinases AC-I and AC-II, and chondroitinase B for analyzing the disaccharides from both the CS and DS chains, the CS moiety, and the DS moiety, respectively [22]. The HPLC data obtained from the digest of the CS preparation from porcine cartilage are summarized in Table 1. The data obtained from the digest with chondroitinase ABC revealed that the non- and low sulfated disaccharides, ΔHexUA-GalNAc (ΔO), ΔHexUA-GalNAc(6-*O*-sulfate) (ΔC), and ΔHexUA-GalNAc(4-*O*-sulfate) (ΔA), where ΔHexUA represents 4-unsaturated hexuronic acid, were major disaccharide units, accounting for ~7, 28, and 64 %, respectively (Table 1), consistent with a recent report [25]. Notably, a small yet appreciable proportion of the disulfated

Table 1 Disaccharide composition of the CS preparation from porcine cartilage. The CS preparation from porcine cartilage was digested with three kinds of chondroitinases and analyzed by anion-exchange HPLC after labeling with a fluorophore 2AB as detailed under "Materials and Methods"

Disaccharide unit	pmol/μg powder (mol %)[b]		
	CS/DS[a]	CS moiety[a]	DS moiety[a]
ΔO[c]: ΔHexUA-GalNAc[d]	60 (7)	76 (9)	N.D.
ΔC: ΔHexUA-GalNAc(6S)	246 (28)	255 (30)	N.D.
ΔA: ΔHexUA-GalNAc(4S)	556 (64)	523 (61)	3 (90)
ΔD: ΔHexUA(2S)-GalNAc(6S)	4 (0.5)	2 (0.2)	N.D.
ΔB: ΔHexUA(2S)-GalNAc(4S)	2 (0.3)	N.D.[e]	0.3 (10)
ΔE: ΔHexUA-GalNAc(4S,6S)	4 (0.4)	4 (0.4)	N.D.
Total	872 (100)	860 (100)	3.3 (100)

[a] The total amount and disaccharide composition of the CS and DS chains, the CS moiety, and the DS moiety were calculated based on the peak area in the chromatograms of digests obtained with chondroitinase ABC, a mixture of chondroitinases AC-I and AC-II, and chondroitinase B, respectively

[b] Values are expressed as pmol of disaccharide per mg of protein in the cells, and calculated based on the peak areas of the disaccharides detected by anion-exchange HPLC (data not shown)

[c] For each disaccharide structure in detail, see Refs. [1] and [4] also

[d] ΔHexUA represents 4-unsaturated hexuronic acid

[e] *N.D.* not detectable (<1 pmol/mg protein)

disaccharides, ΔHexUA-GalNAc(2-,6-*O*-disulfate) (ΔD)(0.5 %), ΔHexUA(2-*O*-sulfate)-GalNAc(4-*O*-sulfate) (ΔB)(0.3 %), and ΔHexUA-GalNAc(4-, 6-*O*-disulfates) (ΔE)(0.4 %), were also detected in the porcine cartilage CS (Table 1). To further determine GlcUA- or IdoUA-containing structures in the CS preparation, an analysis was also carried out using a mixture of chondroitinase AC-I and AC-II, or chondroitinase B specific for GlcUA- or IdoUA-containing structures, respectively [22]. The chondroitinase AC digest showed that CS structures, namely GlcUA-containing disaccharides, were abundant in CS-P (98.6 %). Interestingly, a small but significant amount (0.4 %) of DS-structure also existed in the chondroitinase B digest of CS-P (Table 1).

Kinetic interactions between low sulfated CS/DS and growth factors/cytokines

Highly sulfated CS-E chains rich in E-units strongly interact with heparin-binding proteins such as MK, PTN, FGF2, FGF10, FGF16, FGF18, and heparin-binding epidermal growth factor-like growth factor expressed in the brain [12]. Furthermore, vascular endothelial growth factor, which is a major contributor to angiogenesis, also binds to the CS-E-like structure in tumor vessels [26]. On the other hand, D-unit-containing structures of CS/DS chains from embryonic pig brains and a CS-D preparation from shark fin cartilage can promote neurite outgrowth of embryonic mouse hippocampal neurons in culture through specific interaction with PTN [7, 13].

In this study, the binding of low sulfated CS and DS chains to various growth factors and cytokines was examined by SPR after immobilization of the CS/DS chains at their carboxy groups to the surface of streptavidin-coated sensor chips. The kinetic parameters for the interactions of representative neurotrophic and growth factors, and cytokines with CS variants were determined by varying the concentrations of the individual proteins using SPR as illustrated in Figs. 1, 2, 3, 4, 5 and 6, which show the overlay of the sensorgrams obtained by applying varying concentrations of the proteins to the CS-immobilized sensor chips. The k_a, k_d, and K_D values for each protein were calculated using a BIAevaluation software, and the results are summarized in Tables 2 and 3. Note that no interactions of MIP-1α or MIP-1β with CS/DS were observed (data not shown).

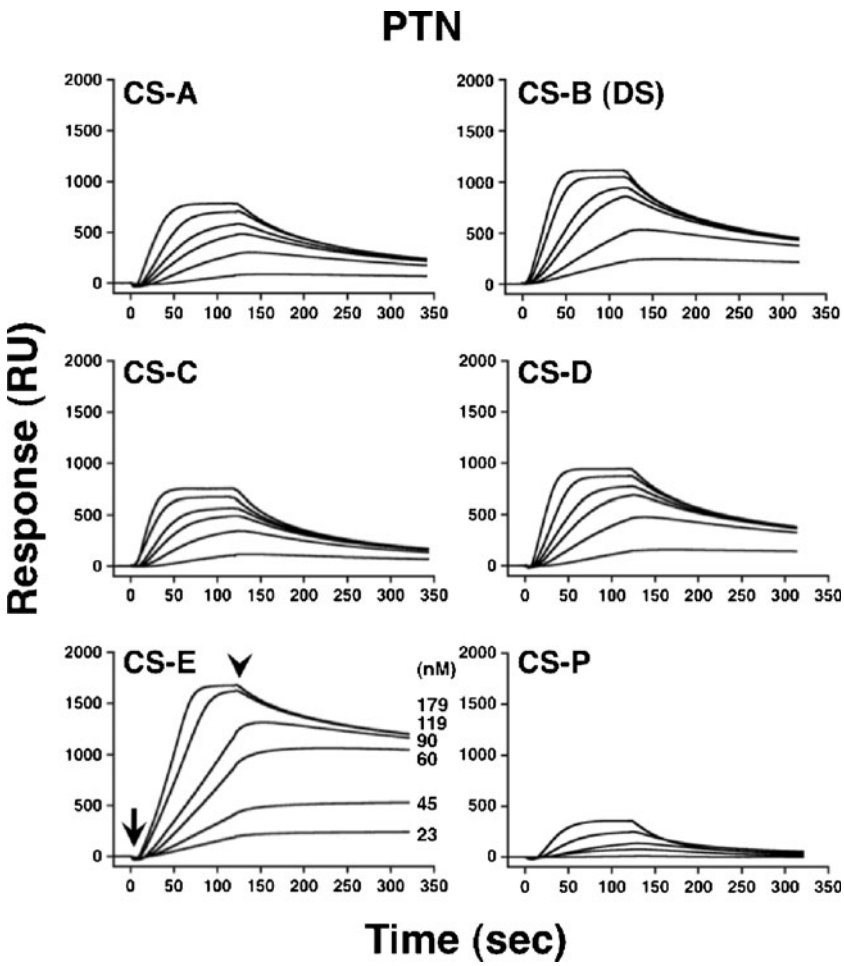

Fig. 1 Sensorgrams for the binding of recombinant PTN to CS variants. Various concentrations, 23–179 nM, of a recombinant PTN were injected onto the surface of the sensor chips immobilized with CS-A, nitrous acid-treated CS-B (DS), CS-C, CS-D, CS-E, and porcine cartilage CS (CS-P). The sensorgrams obtained with each GAG preparation were overlaid in the respective panel using the BIAevaluation software. The *arrow* indicates the beginning of the association phase initiated by the injection of growth factors, and the *arrowhead* indicates the beginning of the dissociation phase initiated with the running buffer (10 mM HEPES, pH7.4/150 mM NaCl/3 mM EDTA/0.005 % Tween20). Interactions of CS-A – CS-E and CS-P with PTN were analyzed by Biacore 2000 and T200, respectively

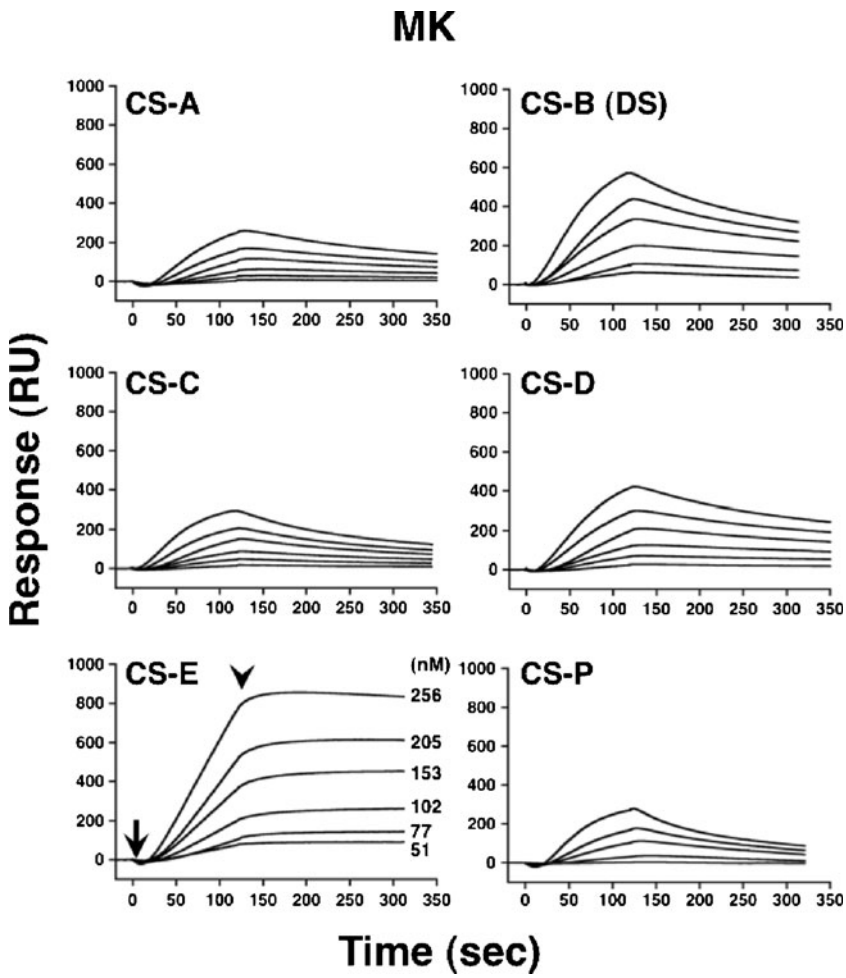

Fig. 2 Sensorgrams for the binding of recombinant MK to CS variants. The binding experiments were carried out as described in the legend to Fig. 1

CS-A, CS-B (DS), and CS-C, which consists of the repeating disaccharides, GlcUA-GalNAc(4-*O*-sulfate), IdoUA-GalNAc(4-*O*-sulfate), and GlcUA-GalNAc(6-*O*-sulfate) as a major disaccharide unit, respectively [17], and are relatively low sulfated, interacted with PTN, MK, and MIG (Figs. 1, 2 and 5) to considerable degrees, where the K_D values obtained using the "1 : 1 binding model with mass transfer" for the binding of each protein to CS-A, DS, and CS-C were comparable (Tables 2 and 3), suggesting that their interactions may be independent of the sulfation position and the type of uronic acids in the low sulfated CS and DS chains. However, a possibility exists that CS/DS chains in addition to HS may have two or more binding sites in the chains interacting with functional proteins [4, 7]. Hence, the kinetics of association and dissociation of various proteins to GAGs were calculated from sensorgrams using the heterogeneous ligand fitting model as well (Table 4). Although the calculated K_D values between individual CS preparations (CS-A, DS, and CS-C) and each growth factor (PTN, MK, or MIG) were slightly higher than those obtained from the "1 : 1 binding model with mass transfer", similar trends were observed for the affinity of individual CS or DS preparations with each protein (Table 4).

CS-A, DS, and CS-C strongly bound RANTES as in the case of CS-E (Fig. 4). Notably, the sensorgrams showed slower association and dissociation rates for all CS variants (Fig. 4). The K_D values obtained from both the "1 : 1 binding model with mass transfer" and "heterogeneous ligand fitting model" were 5~70-fold lower for the binding of RANTES to DS than to CS-A (Tables 3 and 4), suggesting that IdoUA-containing structures may be preferred by RANTES. Furthermore, the K_D value was 9-fold lower for the binding of RANTES to CS-A than CS-C (Table 3). On the other hand, the K_D value from the "heterogeneous ligand fitting model" was 33-fold higher for the binding of RANTES to CS-A than CS-C (Table 4), indicating that the relative importance of 4-*O*- or 6-*O*-sulfation on the GalNAc residues in CS chains for interactions remains to be elucidated.

CS-A and CS-C interacted weakly but significantly with HGF (Fig. 3), whereas the affinity of DS for HGF (K_D= 3.9 nM) was higher than that of CS-A or CS-C (K_D values

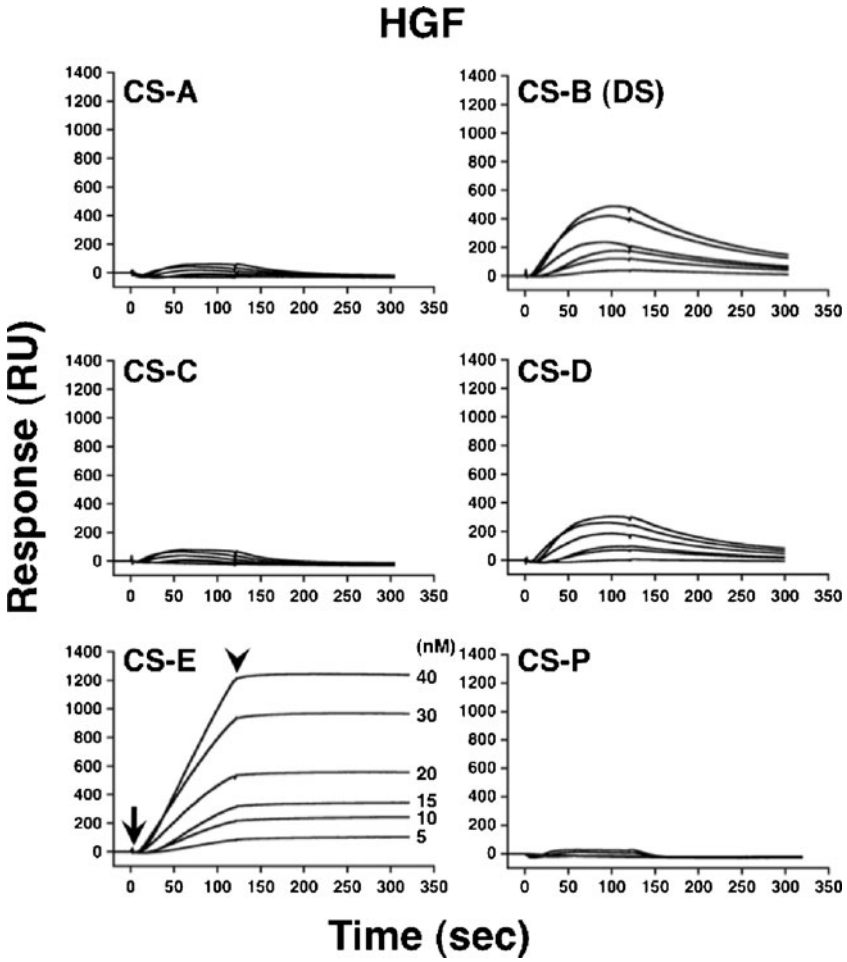

Fig. 3 Sensorgrams for the binding of recombinant HGF to CS variants. The binding experiments were carried out as described in the legend to Fig. 1

from the "1 : 1 binding model with mass transfer" for CS-A and CS-C could not be determined due to faint responses. See Fig. 3) (Table 2), which was consistent with the K_D values obtained according to the "heterogeneous ligand fitting model" (Table 4). In fact, it is known that HGF binds to DS although the interaction is weaker than with heparin [27]. Thus, our observations are well consistent with the previous findings [27].

All the CS variants tested except CS-E showed weak binding comparable to SDF-1β while remarkable interaction of CS-E with the cytokine was observed (Fig. 6) as previously reported [5], indicating that E-units but not the other units are critical for the robust binding.

The CS-P preparation, which contains A- and C-units as major disaccharides (64 and 28 %, respectively) (Table 1), showed high affinity for PTN, MK, and MIG, and moderate affinity for RANTES, but did not bind with HGF or SDF-1β (Figs. 1, 2, 3, 4, 5 and 6; Tables 2, 3 and 4). CS-A and CS-C, which contain A- and C-units as major disaccharides (77 and 20 % or 20 and 70 %, respectively) (Table 6 in ref. 17), also showed similar trends (Tables 2 and 3), implying a possible correlation between fine sequences composed of A- and C-units and their capacity to bind to these proteins.

Interaction kinetics between highly sulfated CS chains and growth factors/cytokines

In the case of CS-E rich in E-disaccharide units, the sensorgrams showed intense interactions with PTN, MK, HGF and SDF-1β as reported previously [5, 12, 27] (Figs. 1 and 2). It was also demonstrated for the first time to our knowledge that the bindings of RANTES and MIG was at the nM level (K_D=47 and 4.9 from the "1 : 1 binding model with mass transfer", respectively), suggesting also higher affinity than for other CS variants (Figs. 3, 4, 5 and 6; Tables 2 and 3). Interestingly, the shape of the dissociation phase of the binding of SDF-1β with CS-E was different from that of the binding of the other proteins with CS-E in that the dissociation rate was higher and reached the base line only after 2 min (Fig. 6), reflecting a fast association and dissociation. The *ka* value for the binding of SDF-1β to CS-E was higher than that of RANTES but lower than that of MK,

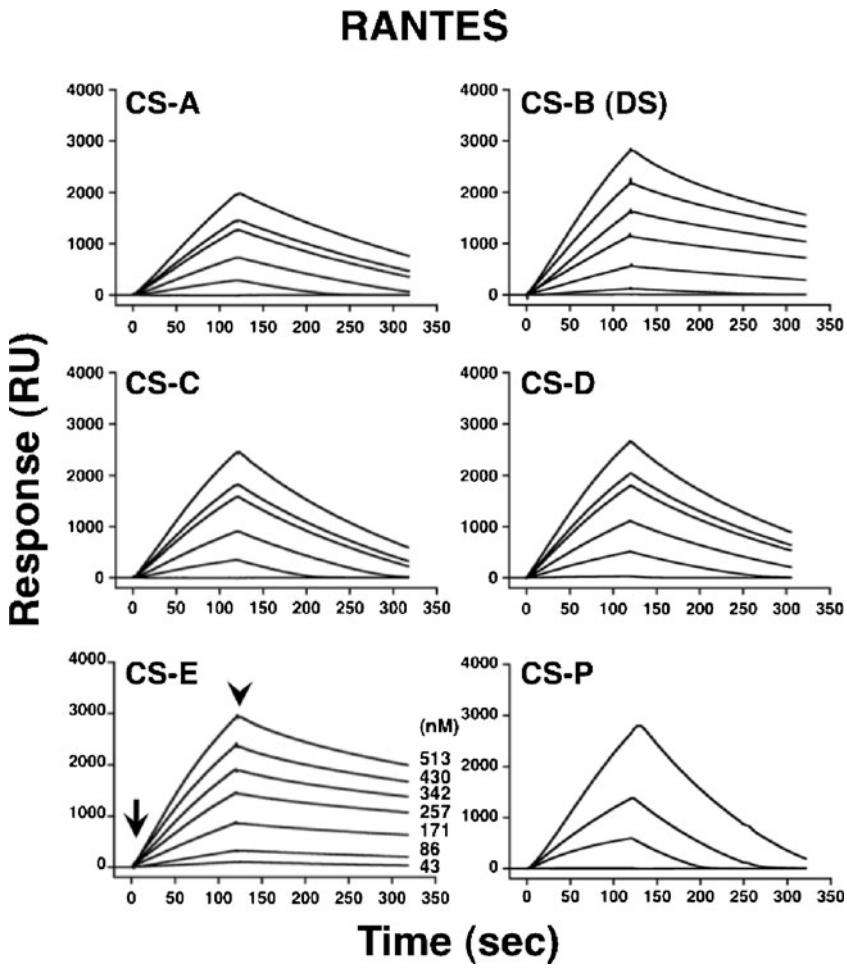

Fig. 4 Sensorgrams for the binding of recombinant RANTES to CS variants. The binding experiments were carried out as described in the legend to Fig. 1

PTN, HGF or MIG (Tables 2 and 3). In strong contrast, the dissociation of PTN and MK was very slow and negligible, respectively (Figs. 1 and 2), as reported previously [12], indicating the fast release of SDF-1β from the target receptor or cells after the binding. SDF-1β is unique in this context as compared with the other proteins tested. Thus, these observations may suggest that SDF-1β specifically recognizes E-units in CS chains, immediately presents the receptor, CXCR4, as previously reported [5, 28] and exerts specific functions in a tissue-dependent manner.

The commercial CS-D preparation, which contains D-units (~30 %) [17], bound PTN, MK, HGF and MIG with high affinity (*Kd* values of 14, 64, 9.2, and 37 nM obtained using the "1 : 1 binding model with mass transfer" or 44, 974, 107, and 142 nM obtained using the "heterogeneous ligand fitting model"), whereas it bound RANTES with low affinity and showed negligible binding to SDF-1β (Tables 2, 3 and 4), implying a possible contribution of D-units to the binding of PTN, MK, HGF, and MIG. In fact, D-unit-containing functional domain structures are crucial to the binding with PTN, its signaling, neuritogenesis [7, 23] and development of Purkinje cells [29, 30].

Discussion

In this study, CS variants were demonstrated to interact with various growth/neurotrophic factors, and cytokines using SPR. Low-sulfated CS-A, DS, and CS-C bound the proteins tested, all of which are so-called heparin-binding proteins [31], with significant affinity (K_D values in the nM range) although their binding was weaker than that with CS-E (Table 2). It should be noted that the SPR binding assays in this study were carried out using the BIAcore systems unlike the "IAsis" system used in the previous study [12]. Presumably, this is why somewhat lower K_D values were obtained in this study, for example, for the binding of CS-E with PTN and MK (11.4 vs 5.5 nM and 61.6 vs 0.13 nM, respectively). The apparent kinetics parameters were calculated by using both the "1 : 1 binding model with mass transfer" and "heterogeneous ligand fitting model". Their

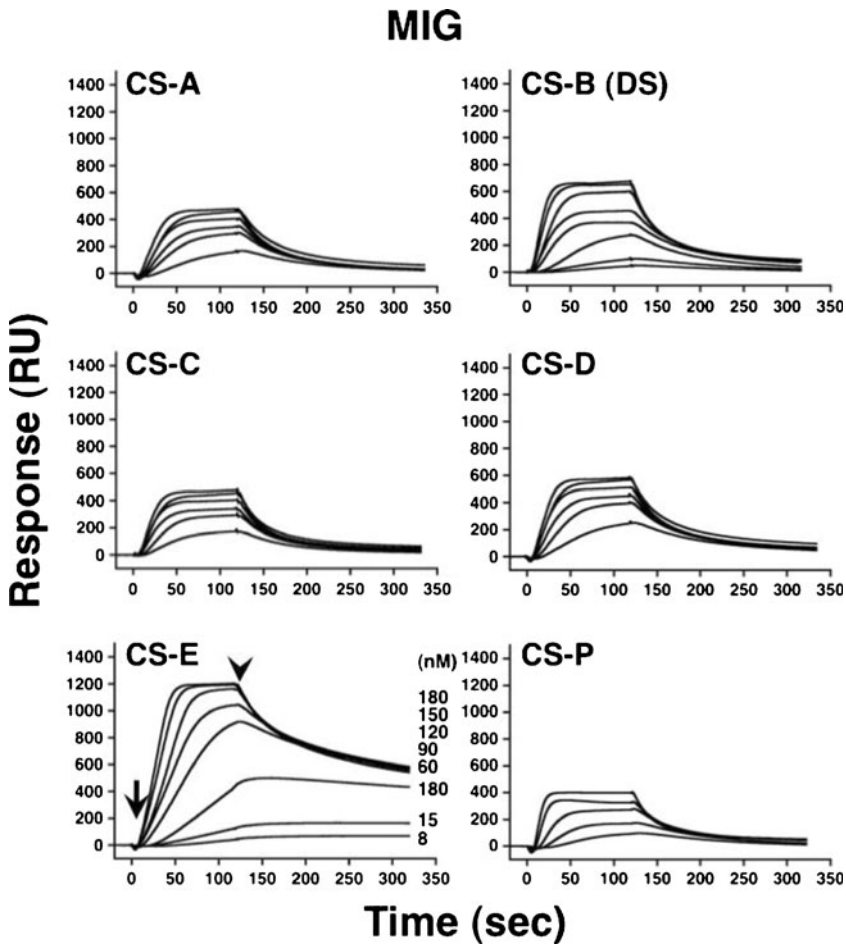

Fig. 5 Sensorgrams for the binding of recombinant MIG to CS variants. The binding experiments were carried out as described in the legend to Fig. 1

apparent K_D values were comparable and showed similar trends in both models. Although a possibility cannot be excluded that the CS/DS chains contain more than two kinds of binding sites with distinct affinities, no fitting model has been available so far to calculate kinetic parameters in such cases.

HS traps various growth factors, morphogens, and effector proteins with high affinity through the domains containing the trisulfated disaccharides in HS chains [32, 33]. Desulfation of 6-*O*-sulfate groups from glucosamine residues in the trisulfated disaccharides by the actions of HS endo-6-*O*-sulfatase (Sulf) results in the low affinity of the domains, which present the proteins to the respective receptor(s) [34]. CS chains may also regulate the effector proteins even through the low sulfated regions rich in A- and/or C-units if a small number of disulfated disaccharides such as B-, D-, or E-units are included. Since the commercial CS-A and CS-C preparations from whale and shark cartilage contain small but significant proportions of D- and/or E-units (0.3–8.5 %, respectively) [Table 6 in 17], the possibility cannot be excluded that D- and/or E-units contained in CS-A or CS-C chains interact with effector proteins. Further analysis is necessary to clarify such a possibility.

We have demonstrated that CS and DS chains derived from various biological sources including embryonic pig brain and porcine fetal membranes in addition to commercial CS and DS preparations, show neurite outgrowth-promoting activity *in vitro* and strong binding to various neurotrophic and growth factors such as PTN, MK, brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor, HGF, FGF-2, -7, -10, -16, -18 using SPR [23, 35–37]. They contain significant proportions of IdoUA-containing structures (DS-structures) in addition to highly sulfated disaccharide units. Thus, CS/DS-hybrid type structures with neuritogenic activity may contain unique sequences with binding activity for neurotrophic factors. It was recently reported that 6-*O*-sulfation of brain CS chains is required for the regulation of the critical period for cortical plasticity [38]. Such an observation may suggest that low sulfated CS-C chains, which interact with PTN, MK, and HGF (Figs. 1, 2 and 3), may also contain unique binding sequences, which are composed of GlcUA- or IdoUA-

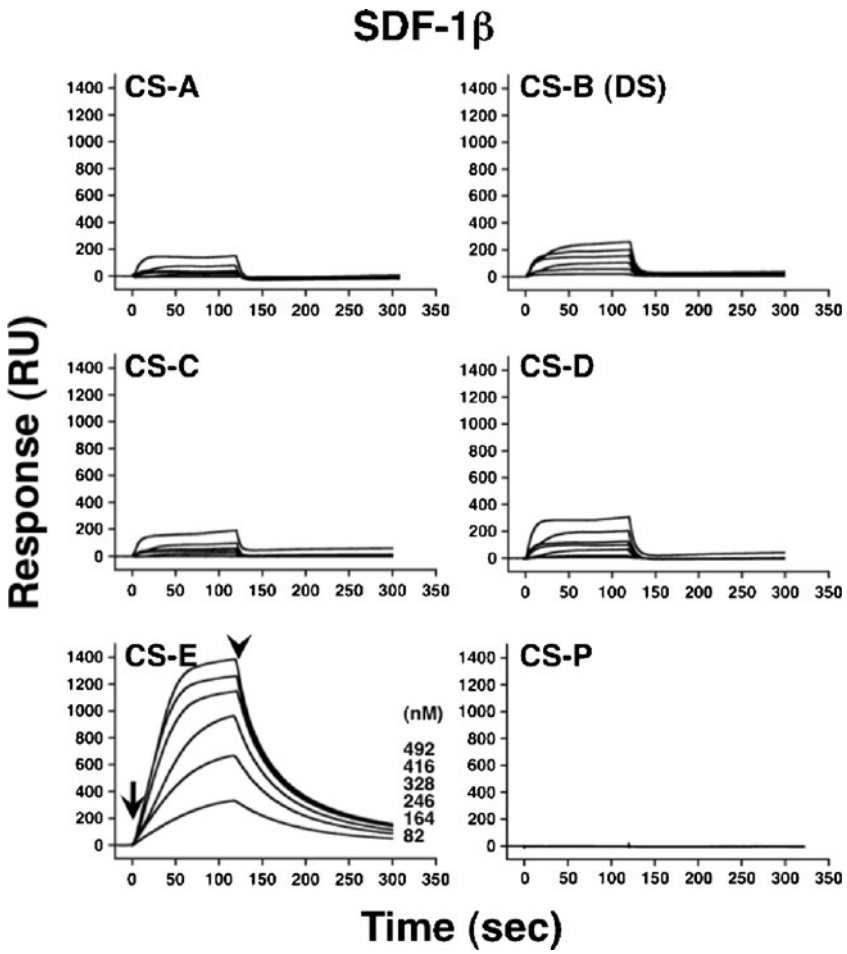

Fig. 6 Sensorgrams for the binding of recombinant SDF-1β to CS variants. The binding experiments were carried out as described in the legend to Fig. 1

containing nonsulfated and monosulfated disaccharide units.

Fukui and co-workers have developed oligosaccharide microarrays, which are useful for assigning the specificity of interactions between carbohydrates including CS/DS and proteins [39], and have reported that CS-A, DS, and CS-C-derived oligosaccharides interact with HGF, FGF7, and RANTES using CS and/or DS 8- or 10-mers [40]. These observations are consistent with our results obtained using SPR (Figs. 3 and 5), although the actual sequences and sulfation patterns of CS and/or DS interacting with the respective proteins remain to be investigated. Recently, we determined the sequences of CS/DS chains interacting with PTN and HGF [7, 41]. Six PTN-binding octasaccharide sequences, ΔC-C-D-C, ΔA-C-D-C (or ΔC-A-D-C), ΔD-C-D-C, ΔC-D-D-C (or ΔC-D-iD-C), and ΔE-D-A-D (or ΔE-D-IA-D), have been isolated from embryonic pig brain [7]. In addition, ΔA-iB-iB, which bound not only PTN but also HGF has been isolated from shark skin, and the sugar sequence, conformation and electrostatic potential distribution of the oligosaccharides were elucidated by a combination of biochemical and computational approaches [41]. PTN is involved in the morphogenesis of Purkinje cells in the cerebellum through binding to CS side chains on PTPζ, which is a receptor type tyrosine phosphatase and a CS-PG [3, 29]. In addition, PTN and D-unit-containing CS chains, which are recognized by the anti-CS-D antibody MO-225 [42, 43], are co-localized in the molecular layer [29]. Furthermore, the *UST* transcript encoding uronosyl 2-*O*-sulfotransferase, which transfers sulfate from 3′-phosphoadenosine 5′-phosphosulfate to the C-2 position of uronic acids in CS/DS chains to form D-units, is expressed in the developing cerebellum [30, 44]. These findings suggest that specific modifications by sulfation in CS chains regulate the neuritogenesis of cerebellar neurons. Hence, in addition to the isolated oligosaccharides interacting with PTN and/or HGF, the identification of the oligosaccharide sequences of other CS/DS-binding proteins would provide a fundamental structural platform for the drug design of sugar or sugar mimetics for neurodegenerative disorders in the long run.

Chemokines are a subset of cytokines, which act on cell surface receptors to regulate cell migration, lymphocyte

Table 2 Kinetic parameters for the interaction of various growth and neurotrophic factors with immobilized CS variants and DS. The k_a, k_d, and K_D values were determined using a 1:1 Langmuir binding model with mass transfer as described under "Materials and Methods". The values for each CS variant are expressed as the mean ± S.E. of five different concentrations

	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D (nM)
PTN			
CS-A[a]	$(3.7\pm0.95)\times10^{6}$	$(7.3\pm2.1)\times10^{-2}$	32±12
CS-B (DS)[a]	$(4.5\pm1.3)\times10^{6}$	$(7.1\pm2.1)\times10^{-2}$	47±31
CS-C[a]	$(5.0\pm1.5)\times10^{7}$	$(9.8\pm3.8)\times10^{-1}$	18±3.2
CS-D[a]	$(1.4\pm0.075)\times10^{7}$	$(2.0\pm0.32)\times10^{-1}$	14±2.5
CS-E[a]	$(1.8\pm0.97)\times10^{6}$	$(1.0\pm0.64)\times10^{-2}$	5.5±3.2
CS-P[a]	$(9.3\pm3.9)\times10^{4}$	$(9.5\pm2.7)\times10^{-3}$	50±40
MK			
CS-A	$(2.1\pm0.46)\times10^{4}$	$(2.5\pm0.42)\times10^{-3}$	177±73
CS-B (DS)	$(4.4\pm1.9)\times10^{4}$	$(2.3\pm0.57)\times10^{-3}$	159±84
CS-C	$(3.6\pm1.4)\times10^{5}$	$(1.4\pm0.55)\times10^{-2}$	47±7.0
CS-D	$(8.3\pm3.5)\times10^{4}$	$(2.2\pm0.82)\times10^{-3}$	64±36
CS-E	$(2.7\pm0.34)\times10^{5}$	$(2.6\pm0.79)\times10^{-5}$	0.13±0.065
CS-P	$(3.3\pm0.7)\times10^{3}$	$(4.8\pm1.1)\times10^{-3}$	1,940±646
HGF			
CS-A	N.D.[b]	N.D.	—[c]
CS-B (DS)	$(2.7\pm0.90)\times10^{7}$	$(8.0\pm2.1)\times10^{-2}$	3.9±1.3
CS-C	N.D.	N.D.	—
CS-D	$(1.4\pm0.40)\times10^{6}$	$(1.2\pm0.35)\times10^{-2}$	9.2±3.0
CS-E	$(7.2\pm1.9)\times10^{5}$	$(1.7\pm1.5)\times10^{-4}$	0.17±0.10
CS-P	N.D.	N.D.	—

[a] CS-A from whale cartilage; nitrous acid-treated CS-B (DS) from porcine skin; CS-C from shark cartilage; CS-D from shark fin cartilage; CS-E from squid cartilage; and CS-P from porcine cartilage

[b] *N.D.* not determined due to feeble interaction (*see* Fig. 3)

[c] —, not occurring

Table 3 Kinetic parameters for the interaction of cytokines with immobilized CS variants and DS. The k_a, k_d, and K_D values were determined using a 1:1 Langmuir binding model with mass transfer as described under "Materials and Methods". The values for each CS variant are expressed as the mean ± S.E. of five different concentrations

	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D (nM)
RANTES			
CS-A[a]	$(5.9\pm0.74)\times10^{5}$	$(4.4\pm0.21)\times10^{-2}$	882±511
CS-B (DS)[a]	$(5.7\pm1.6)\times10^{4}$	$(7.7\pm2.9)\times10^{-3}$	166±48
CS-C[a]	$(1.7\pm1.0)\times10^{4}$	$(9.8\pm3.1)\times10^{-3}$	7,720±3,100
CS-D[a]	$(1.9\pm0.78)\times10^{4}$	$(1.4\pm0.36)\times10^{-2}$	1,410±370
CS-E[a]	$(8.4\pm1.9)\times10^{4}$	$(3.6\pm0.72)\times10^{-3}$	47±11
CS-P[a]	$(6.7\pm6.5)\times10^{3}$	$(6.0\pm2.6)\times10^{-3}$	26±15 (μM)
MIG			
CS-A	$(9.7\pm2.3)\times10^{5}$	$(5.6\pm1.2)\times10^{-2}$	58±1.1
CS-B (DS)	$(4.2\pm0.45)\times10^{6}$	$(1.8\pm0.41)\times10^{-1}$	42±7.4
CS-C	$(7.0\pm0.89)\times10^{6}$	$(4.7\pm0.80)\times10^{-1}$	64±5.3
CS-D	$(9.7\pm8.5)\times10^{8}$	$(3.4\pm3.1)\times10^{1}$	37±2.3
CS-E	$(3.0\pm1.2)\times10^{6}$	$(2.5\pm1.4)\times10^{-2}$	4.9±2.2
CS-P	$(5.1\pm0.8)\times10^{5}$	$(1.5\pm0.03)\times10^{-2}$	31±4.5
SDF-1β			
CS-A	N.D.[b]	N.D.	—[c]
CS-B (DS)	N.D.	N.D.	—
CS-C	N.D.	N.D.	—
CS-D	N.D.	N.D.	—
CS-E	$(1.4\pm0.78)\times10^{5}$	$(4.0\pm1.4)\times10^{-2}$	908±373
CS-P	N.D.	N.D.	—

[a] CS-A from whale cartilage; nitrous acid-treated CS-B (DS) from porcine skin; CS-C from shark cartilage; CS-D from shark fin cartilage; CS-E from squid cartilage; CS-P from porcine cartilage

[b] *N.D.* not determined due to feeble interaction (*see* Fig. 6)

[c] —, not occurring

homing, inflammation, and wound repair [45]. BBXB and BBBXXB, where B and X stand for a basic and any amino acid, respectively, are common heparin-binding motifs for specific proteins [46]. RANTES is a small protein composed of 68 amino acids, induces leukocyte migration by binding to specific receptors, and exerts HIV suppressive activity [47]. The amino acid sequence of RANTES indicates that it contains two clusters of basic residues [48]. It has been reported that the order of preferences by RANTES interacting with CS/DS is CS-C, DS and CS-A [49]. The present study further defined the binding preferences: CS-E >> DS (CS-B) > CS-A > CS-D > CS-C (Table 3), whose order is different from the previously reported observation [49]. The differences may partly depend on differences in the disaccharide sequences of the preparations used in the previous and present studies. Together, fine sequences of CS/DS interacting with RANTES in leukocytes may provide information on novel drug targets for anti-inflammatory or anti-HIV agents.

MIG is produced by monocytes, macrophages, and endothelial cells through stimulation by interferon-γ, and functions as an inhibitor of tumor growth, angiogenesis and the formation of colonies by hematopoietic progenitors [50–52]. All the types of CS/DS chains tested bound to MIG with high affinity (K_D values in the nM range) with CS-E having the highest affinity (Fig. 5 and Table 3).

The proinflammatory cytokine SDF-1 chemoattracts B and T cells, induces the migration of $CD34^{+}$ stem cells, and inhibits the infection of HIV [53]. Strong interaction of CS-E with SDF-1β was confirmed, unlike for other CS/DS chains (Fig. 6), as reported previously [5]. Hence, E-unit-containing structures may be involved in the exertion and regulation of CS functions in inflammation and HIV-infection. Further investigation is warranted.

Table 4 Kinetic parameters for the interaction of neurotrophic factors and cytokines with immobilized CS variants and DS using a heterogeneous binding model. The K_D values were determined using a heterogeneous binding model, which can be applied to chains containing two binding sites with different affinities, but not to chains containing three or more binding sites according to the manufacture's instruction. The values for each CS variant are expressed as the mean ± S.E. of four different concentrations

	K_{D1} (nM)[b]	K_{D2} (nM)
PTN		
CS-A[a]	32±14	37±13
CS-B (DS)[a]	225±124	251±113
CS-C[a]	56±31	57±30
CS-D[a]	24±14	44±19
CS-E[a]	69±60	58±50
CS-P[a]	115±47	110±53
MK		
CS-A	371±231	639±431
CS-B (DS)	347±205	329±143
CS-C	1,050±705	783±452
CS-D	701±415	974±605
CS-E	0.3±0.08	0.3±0.08
CS-P	491±256	807±405
HGF		
CS-A	N.D.	N.D.
CS-B (DS)	54±27	63±23
CS-C	N.D.	N.D.
CS-D	114±42	107±46
CS-E	0.7±0.5	0.7±0.5
CS-P	N.D.	N.D.
RANTES		
CS-A	7±1 (μM)	69±55 (μM)
CS-B (DS)	2±0.5 (μM)	1±0.3 (μM)
CS-C	2±0.4 (μM)	3±0.9 (μM)
CS-D	1±0.3 (μM)	2±0.6 (μM)
CS-E	0.2±0.005 (μM)	0.3±0.01 (μM)
CS-P	12±10 (μM)	19±15 (μM)
MIG		
CS-A	144±41	144±43
CS-B (DS)	636±294	494±229
CS-C	85±25	90±22
CS-D	212±46	142±34
CS-E	16±14	92±62
CS-P	64±27	62±27
SDF-1β		
CS-A	N.D.	N.D.
CS-B (DS)	N.D.	N.D.
CS-C	N.D.	N.D.
CS-D	N.D.	N.D.
CS-E	284±154	338±130
CS-P	N.D.	N.D.

[a] CS-A from whale cartilage; nitrous acid-treated CS-B (DS) from porcine skin; CS-C from shark cartilage; CS-D from shark fin cartilage; CS-E from squid cartilage; CS-P from porcine cartilage

[b] K_{D1} and K_{D2} values represent equilibrium dissociation constants for the first and second binding sites in CS/DS chains, respectively ($K_D=kd/ka$)

[c] *N.D.* not determined due to feeble interaction

Taken together, our results suggest that low sulfated CS-A, DS (CS-B), and CS-C in addition to highly sulfated CS-E may also interact under physiological and pathological conditions with neurotrophic factors, growth factors and/or chemokines, although the interactions appear to be much weaker. In addition, it was revealed that CS-E possesses high affinity for chemokines, MIG and SDF-1β. Further structural elucidation of the functional domains in the CS and DS chains interacting with these proteins, and subsequent computer modeling of such oligosaccharides and their binding sites on the proteins, would provide insights into the functional roles of CS/DS chains and novel drug designs.

There have been a number of reports on the biological activities of orally administered CS preparations, including anti-inflammatory and chondro-protective properties [54–56], while no significant or clinically important effect towards the pain in osteoarthritis has been observed [57]. Thus, the efficacy of CS to reduce pain in osteoarthritis is still a matter of controversy. While polysaccharides such as CS are poorly absorbed through the digestive system, it has been reported that a chemically produced low molecular weight CS preparation showed preventive effects on type II collagen-induced arthritis in DBA/1J mice and better permeability through Caco-2 cells [58], suggesting that low molecular weight CS is absorbed more efficiently. Therefore, absorption of CS may largely depend on the degradation by colon bacteria [59, 60]. The chondroprotective activities of orally administered CS have been proposed to result from an increase in the biosynthesis of connective tissue components, such as hyaluronan, at disease sites [61]. Both *in vitro* and *in vivo* studies have shown that CS regulates the formation of new cartilage by stimulating the synthesis of collagen, proteoglycans, and hyaluronan by chondrocytes [62]. Interestingly, orally administered CS inhibits the IgE-mediated allergic response by down-regulating Th2 responses in mice [63].

Thus, the present study has revealed that relatively low sulfated CS, DS, or hybrid chains from several biological sources can interact with various effector proteins including growth factors and a proinflammatory cytokine with significant affinity, implying that these chains may be useful if their absorption efficiency is improved for example by fragmentation or chemical modification.

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