Research Article

Effect of Molecular Sizes of Chondroitin Sulfate on Interaction with L-Selectin

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Chondroitin sulfate (CS) is a glycosaminoglycan (GAG) side chain of proteoglycans (PGs) which are widely distributed in the extracellular matrix and at cell surface. CS shows a highly structural diversity in not only molecular weight (MW) but sulfonation pattern. CS has been reported to exert anti-inflammatory activity by having effects on cytokine production by helper T cells. In this study, we focused on the structures of CS chains, especially MW of CS, and investigated effect of the different MW of CS on binding affinity with L-selectin and cytokine production by murine splenocytes. Firstly, we fractionated CS by employing gel filtration chromatography and obtained several CS fractions with different MW. Then the interaction between fractionated CS and L-selectin was analyzed by surface plasmon resonance (SPR). Finally, the influence of MW of CS on cytokine production by murine splenocytes was investigated *in vitro*. The results showed that interferon-gamma production was significantly increased by mouse splenocytes cocultivated with CS. On the contrary, CS inhibited interleukin 5 production by murine splenocytes depending on MW of the cocultivated CS. These results strongly indicate the existence of the optimal molecular size for an anti-inflammatory effect of CS through cytokine production by murine splenocytes.

1. Introduction

Chondroitin sulfate (CS) belongs to a family of glycosaminoglycans (GAG), which is a linear, sulfonated polysaccharide, such as heparin (HP), heparan sulfate (HS), and dermatan sulfate (DS). CS is composed of a repeating disaccharide unit, [-4] GlcA (β1-3) GalNAc (β1-]$_n$, where GlcA is glucuronate and GalNAc is *N*-acetylgalactosamine [1]. CS chains show structural diversity because sulfonate groups can be bound to variety positions of the disaccharide units, and its molecular weight (MW) is also highly variable due to different numbers of the disaccharide unit [2–4]. On the other hand, it is well known that CS and other GAGs form conjugates with the specific proteins called proteoglycans (PGs) and PGs are ubiquitously distributed in not only extracellular matrix but also on cell surface in animal tissues. PGs are thought to be participating in cell-cell communications such as a cell adhesion and signalling events *via* proteins, especially; HS chains can control a signalling by interacting with cell growth factors and HP has been widely used as an anticoagulant agent [5–7]. Correspondingly, CS has also a similar function and it has been reported that CS contributes to various bioactivities, for example, development of central nervous system, wound healing, and signalling via cell growth factors [8–11]. Furthermore, there have been a large number of reports on anti-inflammatory activity of CS [12–14]. Kawashima et al. revealed that versican, a large CSPG, in the kidney reacts with leukocyte adhesion molecule, L-selectin, by interacting with CS chains, and the binding affinity of CS against L-selectin differs with the sulfonate positions of CS chains [15–18].

Generally, immune response is classified into two types, innate and adaptive immunity systems. Innate immunity system works as the first defense against invading pathogens and then differentiated lymphocytes, for example, killer and

helper T cells, and antibody-secreting cells, play a role in antigen-specific immune response. The encounter with antigen can induce the differentiation of helper T cells to at least two functional classes of cell types, Th1 cells and Th2 cells, which secrete specific cytokines such as interferon-gamme (IFN-γ) or interluekin-4 (IL-4), respectively [19, 20]. Once an undifferentiated T cell called naive T cell begins to develop along with one pathway, it tends to become progressively polarized in that direction. This polarization is regulated by cytokines which Th1 cell or Th2 cell secreted. We have already reported that CS upregulates the antigen-specific Th1 immune response on murine splenocytes sensitized with ovalbumin (OVA) *in vitro*, and that CS suppresses the antigen-specific IgE responses [12]. Moreover, we showed that the content, position, and number of *O*-sulfo groups in CS are of importance in immunological activity *in vitro* [13]. Furthermore, Th1-promoted and Th2-inhibitory activity of CS on murine splenocytes could be associated with binding to L-selectin [13]. These findings might have been clarified the contribution of *O*-sulfo groups to immunological activity of CS *via* L-selectin.

In this study, we have further investigated the effect of MW of CS on the immune response, especially cytokine production by murine splenocytes sensitized with OVA, *in vitro*.

2. Materials and Methods

2.1. Chemicals. CS-A, prepared from bovine tracheal cartilage and having an average molecular weight of 15 kDa, was a gift from Shin-Nippon Yakugyo Co. (Tokyo, Japan). CS-C was kindly provided by San-Ei Gen FFI Co. (Osaka, Japan). CS-A from whale cartilage having an average molecule weight of 21 kDa was purchased from Seikagaku Kogyo Co. (Tokyo, Japan). CS-B, CS-D, CS-E, unsaturated disaccharides, and chondroitinase ABC were also purchased from Seikagaku Kogyo Co. (Tokyo, Japan).

2.2. Preparation of Fractionated CS. Solution of CS (10 mg/mL) dissolved in purified water was fractionated by using gel filtration column (100 cm × 4.6 cm i.d.) filled with Sephadex G-100 superfine (GE Healthcare, UK). The mobile phase consisted of 0.2 M NaCl. One hundred drops of eluate solution were collected in each glass vial. Then fractions were detected by UV absorbance at 204 nm. Twenty fractions were collected in one and concentrated into one-twentieth by evaporation followed by desalting on a HiTrap Desalting Column.

The separation of desalted fractionated CS was confirmed by using gradient polyacrylamide gel electrophoresis (PAGE). Ten μg of fractionated CS was dissolved in 50 μL of 50% sucrose solution containing 0.01% bromophenol blue and loaded on a gradient PAGE gel (PAGEL NPG-1020 L, 10–20%, 90 mm × 78 mm × 1 mm gel thickness, ATTO Co. Ltd., Tokyo). The electrophoresis was conducted by using a 0.2 M tris-1.2 M glycine mixture as a buffer under 100 V for 60 min.

The molecular weight of each fractionated CS was determined with a high-performance gel chromatography.

The average molecular weight of fractionated CS was calculated from standard curve prepared by using molecular weight known CS sample. The structures of fractionated CS were confirmed by ^{1}H NMR (500 MHz) [21].

2.3. Disaccharide and Composition Analyses of Fractionated CS. The determination of unsaturated disaccharides (ΔDi-0S, ΔDi-4S, ΔDi-6S, ΔDi-diSE, ΔDi-diSB, ΔDi-diSD, and ΔDi-triS), prepared from the fractionated CS, was performed on the lyase-digested samples using high-performance liquid chromatography [22, 23]. Briefly, chondroitinase digestion was performed using tris-acetate buffer (5 μL, 0.2 M, pH 8.0) and 10 μL of an aqueous solution containing chondroitinase ABC (50 mIU) and chondroitinase ACII (50 mIU) were added to a 20 μL portion of the sample solution and incubated at 37°C for 3 h. An 8 μL portion of this mixture was analyzed by high-performance liquid chromatography.

2.4. Estimation of the Molecular Weight of CS. The molecular weights of the fractionated CS were estimated by an HPSEC system that consisted of a Hitachi L-6000 pump (Hitachi Co., Japan), Rheodyne 7725i loop injector (IDEX Health & Science, USA), and a YRD-880 refractive index detector (Shimamura Instruments Co., Japan). The column used was an Asahipak 510 HQ column (7.6 mm, i.d. × 300 mm) (Shodex, Showa Denko Co. Ltd., Tokyo) and eluted with 10 mM NH$_4$HCO$_3$ at a flow rate of 0.3 mL/min. The molecular weight standards of CS (15.4, 22.4, 31.0, and 50.0 kDa from shark cartilage) were kindly gifted from Seikagaku Co. Ltd. (Tokyo).

2.5. Kinetic Analysis Using BIAcore between CS and L-Selectin. Recombinant mouse L-selectin was purchased from Sigma-Aldrich Japan (Tokyo, Japan). The interaction between fractionated CS and L-selectin was analyzed by a BIAcore biosensor (GE healthcare, Tokyo). All experiments were performed at 25°C. The running buffer which was provided from the manufacture consisted of 10 mM HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% Surfactant P20. First, L-selectin was covalently immobilized on the CM5 sensor chip covered with carboxymethyl dextran via its primary amine groups using an amino coupling kit according to the instructions provided from the manufacturer. The remaining activated groups were blocked with 1 M ethanolamine. One control cell was prepared by blocking with ethanolamine. CS samples were dissolved in 50 mM tris-HCl buffer. The different concentrations of the each fractionated CS were injected at 20 μL/min. The sensor chip surface was regenerated with 1 M NaCl. Affinity kinetic parameters were determined with BIAevaluation 3.0 software using a single-site binding model.

2.6. Animals. Inbred specific pathogen-free BALB/c mice (female, 6 weeks of age) were purchased from Nihon SLC Inc. (Shizuoka, Japan). The mice were maintained in a temperature (25°C), humidity (55%), and light-controlled environment with free access to Rodent diet EQ (Nihon SLC Inc., Shizuoka, Japan) and water. They were acclimatized for at least 1 week before the start of study.
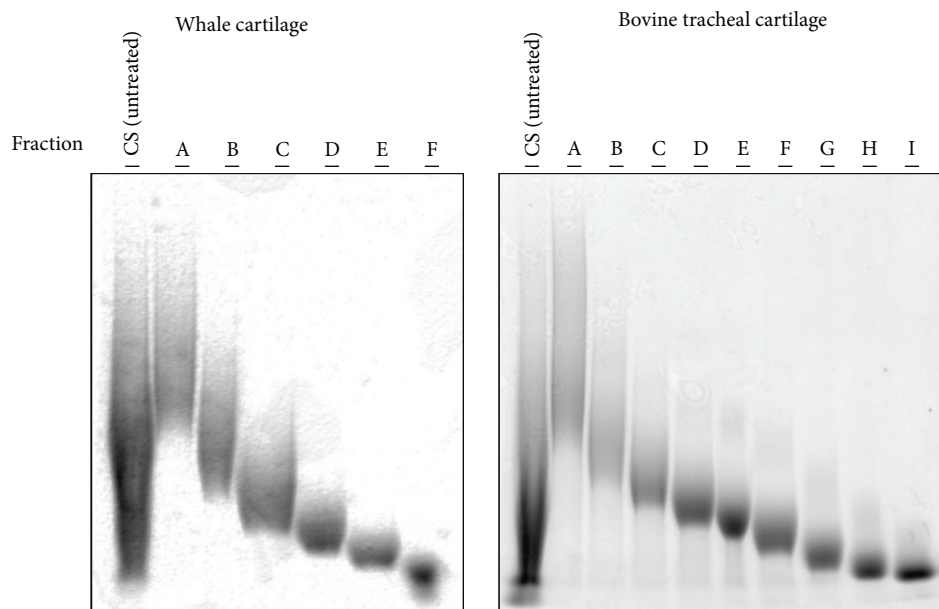
FIGURE 1: Gradient PAGE profiles of original and fractionated chondroitin sulfate.

2.7. Sensitization Protocol. The mice ($n = 5$) were intraperitoneally sensitized with 20 mg of OVA and 2 mg of an aluminium hydroxide hydrate [Al $(OH)_3 \cdot x H_2O$] gel suspension (LSL Co. Ltd., Japan) in a total volume of 400 μL of saline. On 14th day after the first sensitization, the mice were boosted using the same doses of the antigen. On the following day, the mice were sacrificed and their spleens were harvested.

2.8. Preparation and Stimulation of Murine Splenocytes In Vitro. The spleens of mice were removed and cell suspensions were made by passing them through a sterile cell strainer (Becton Dikcinson, CA, USA). The cell suspensions were washed twice in RPMI 1640 medium supplemented with penicillin-streptomycin, 0.1 M sodium pyruvate, 0.2 M L-glutamine, 0.05 mM 2-mercaptoethanol, and FBS, counted by Trypan Blue exclusion staining and adjusted to 5×10^6 cells/mL. The cells were plated at 2 mL/well in a 24-well cell culture cluster (Corning, NY, USA) and challenged with OVA at a final concentration of 100 μg/mL. Furthermore, the samples (each at a final concentration of 0.5 μM) or saline *in vitro* was added to the 24-well culture cluster at 37°C in triplicate in a CO_2 incubator for 72 hrs. The supernatants were then harvested for cytokine production analysis after 72 hrs of incubation.

2.9. Production of Cytokine In Vitro. The cytokines examined in this study were IFN-γ, IL-2, IL-5, and IL-10. The amount of several cytokines in the culture medium after incubation was measured using an ELISA kit (OptEIA TM set, Pharmingen, San Diego, CA, USA). The principle of all ELISA kits was to employ the quantitative sandwich enzyme immunoassay technique. The absorbance was measured at 450 nm with UV-VIS microplate reader (SUNRISE THERMO, TECAN Japan Co. Ltd., Tokyo).

3. Results

3.1. Fractionation of CS. CS has several isomers which have different degrees of sulfonation and binding sites of sulfonate groups [24–26]. Generally, intact CS chains show various MW and a range of their MW is extremely wide from thousands to hundreds of thousands [2–4]. Based on this fact, we firstly fractionated commercially available CS by low-pressure gel chromatography to prepare CS within a strictly limited range of average MW. Two different types of CS samples were applied: one was derived from whale cartilage which is mainly composed of CS-A structure [GlcA (β1-3) GalNAc4S], and the other was from bovine tracheal cartilage which is mainly composed of CS-A structure [GlcA (β1-3) GalNAc4S] [27, 28]. Sephadex G100 Superfine, by which the available fractionation range is from 1 to 100 kDa, was employed for preparation of CS as the average MW of CS from whale cartilage and bovine tracheal cartilage was 21 kDa and 15 kDa, respectively. The structure of each fractionated CS was confirmed by the HPLC for disaccharide analysis and ^{1}H NMR (data not shown). The extent of fractionation of CS was confirmed by applying gradient polyacrylamide gel electrophoresis (PAGE), and we confirmed that we could obtain CS with strictly limited range of MW (Figure 1 and Table 1). Six fractionated CSs from whale cartilage, of which original average MW was 21 kDa, were estimated from 7 to 29 kDa. As for CS from bovine tracheal cartilage, the range of MW was from 6 to 33 kDa.

3.2. Disaccharide Analysis for Fractionated CS. Since it has already been reported that the difference of sulfonation pattern/degree has an impact on a bioactivity of CS, we analyzed the disaccharide composition of the fractionated CS chains. To be concrete, unfractionated and fractionated CSs were degraded by the action of chondroitin lyases and then

TABLE 1: Molecular weight of fractionated chondroitin sulfate.

Fraction	Mean molecular weight (kDa)	
	Whale cartilage	Bovine tracheal cartilage
CS (untreated)	21	15
A	29	33
B	21	26
C	15	21
D	12	17
E	9	15
F	7	13
G	—	9
H	—	7
I	—	6

the compositions of these disaccharides were determined by the reverse-phase ion-pair chromatography with the postcolumn fluorogenic reactions. As a result, it was revealed that the contents of major disaccharide units, which are ΔDi-4S and ΔDi-6S, were quite similar as that of the original CS after fractionation (Table 2). The contents of ΔDi-4S were three and two times higher than those of ΔDi-6S in whale cartilage and bovine tracheal cartilage, respectively. In order to compare the immunoreactivity of both CS from whale and bovine cartilage, 7, 15, and 21 kDa fractionated CS were used for further investigation.

3.3. Binding of CS to L-Selectin.

To investigate the binding affinity between CS and L-selectin, and the effect of MW of CS on the binding affinity with L-selectin, we analyzed the binding affinity between CS and L-selectin by surface plasmon resonance (SPR) measurement employed with BIAcore, which is able to detect a biomolecular interaction without labels and analyzes an affinity and kinetics parameters quantitatively in real time. Diluted commercially available recombinant murine L-selectin in 10 mM acetic buffer (pH 4.0) was immobilized to a CM5 sensor chip by the amino-coupling reaction and 11.7 ng/mm^2 of the L-selectin could be immobilized. Subsequently, the interaction between the fractionated CS and L-selectin was investigated. Figure 2 shows both untreated and fractionated CS could interact with L-selectin through dose dependence. As compared with the association and dissociation phases of sensorgrams of the fractionated CS from bovine tracheal cartilage with L-selectin, those of CS from whale cartilage were steeper; that is, the interaction with CS from whale cartilage with L-selectin is tighter. Kinetic parameters such as association constant (K_A), dissociation constant (K_D), association rate constant (k_a), and dissociation rate constant (k_d) were calculated as the Langmuir formula. Evaluation of the kinetic parameters (Table 3) indicated that K_D of the fractionated CS with 21 kDa in both CS was the smallest and there was the tendency of K_D to increase in a molecular weight-dependent manner. Although untreated CS and fractionated CS had the same average MW, K_D of fractionated CS was smaller in comparison to that of untreated CS. It suggests that CS with narrower molecular distribution has a higher affinity

with L-selectin compared to CS with a wide range of MW. Furthermore, there was considerable difference for untreated CS from whale cartilage and bovine tracheal cartilage in the affinity with L-selectin, even though the disaccharide compositions of these CS samples showed no significant difference (Tables 2 and 3). These results suggest that not only sulfonation pattern including disaccharide sequence but also MW of CS may affect the affinity with L-selectin.

The many studies on bioactivity of CS have been focused on its sulfonation to date. However, our findings suggest that MW of CS could have effect on such a bioactivity of CS as well as sulfonation pattern of CS.

3.4. Effects of Fractionated CS on Cytokine Production of Mice Splenocytes.

To examine the effects of CS with various MWs on cytokine production, splenocytes from OVA-sensitized BALB/c mice were challenged with OVA in the presence and absence of untreated and fractionated CS, and the cytokine levels in the medium of the cultured cells were measured. As shown in Figure 3(a), the levels of IFN-γ, Th1-type cytokine, produced by the splenocytes cultured with fractionated CS from whale cartilage were significantly higher than the levels produced by splenocytes cultured with OVA alone and untreated CS. The production of IFN-γ was the most strongly induced by being cultured with fractionated CS with 21 kDa. As for fractionated CS from bovine tracheal cartilage, it has a tendency to increase the level of IFN-γ in MW dependent. These results coincided with the result of study on the interaction between CS and L-selectin. IL-2 also belongs to Th1-type cytokines but no significant effect on IL-2 production like an IFN-γ production was observed in both CS from whale cartilage and bovine tracheal cartilage (data not shown). In contrast to Th1 cytokines, as shown in Figure 3(b), the levels of Th2 cytokine, IL-5 produced by the splenocytes cultured with fractionated CS from bovine tracheal cartilage were significantly lower than the levels produced by splenocytes cultured with OVA alone. There was a weak tendency to decrease the level of IL-5 production in CS from whale cartilage-treated splenocytes. It seems that fractionated CS has stronger effect on the inhibition of IL-5 production compared to untreated CS. As for the production of IL-10 which is also Th2-type cytokine, decreasing in its production level produced by the splenocytes cultured with fractionated CS was observed (data not shown).

4. Discussion

Selectin is one of cell surface receptors which is engaged in the adhesion between white blood cells and vascular endothelial cells, and it is involved with penetration of white blood cells into extravascular as an adhesion molecule [26]. Presently, three types of selectins, that is, E-, P-, and L-selectin, are reported. E- and P-selectins are expressed in vascular endothelial cell surface and platelets, respectively, and L-selectin is expressed on cell membranes of monocytes, neutrophils, part of natural killer cells, and T and B lymphocytes. Selectins are known as a type I membrane glycoprotein with a C-type lectin domain in its N-terminal domain [29, 30].

TABLE 2: Disaccharide compositions of unfractionated and fractionated chondroitin sulfate samples from whale and bovine cartilage.

Fraction	ΔDi-0S	ΔDi-4S	ΔDi-6S	ΔDi-diS$_E$	ΔDi-diS$_B$	ΔDi-diS$_D$	ΔDi-triS
				Whale cartilage			
CS (untreated)	1.2	76.8	20.9	0.3	N.D.	0.7	Trace
A	1.2	75.7	22.8	0.1	N.D.	0.2	Trace
B	1.3	75.4	22.7	0.2	N.D.	0.4	Trace
C	1.5	75.7	22.4	0.1	N.D.	0.3	Trace
D	1.5	76.3	21.9	0.1	N.D.	0.2	Trace
E	1.5	76.7	21.0	0.2	N.D.	0.5	Trace
F	3.4	76.0	20.4	0.1	N.D.	0.1	Trace
				Bovine tracheal cartilage			
CS (untreated)	8.1	56.5	33.8	0.6	N.D.	1.0	Trace
A	6.7	61.3	30.9	0.5	N.D.	0.7	Trace
B	7.5	62.3	29.0	0.5	N.D.	0.7	Trace
C	7.3	61.7	29.7	0.5	N.D.	0.8	Trace
D	6.6	59.1	33.1	0.4	N.D.	0.7	Trace
E	6.4	55.7	36.9	0.3	N.D.	0.6	Trace
F	6.7	52.1	40.3	0.3	N.D.	0.6	Trace
G	5.4	50.0	43.4	0.4	N.D.	0.8	Trace
H	8.6	50.1	40.2	0.3	N.D.	0.9	Trace
I	8.2	48.7	41.6	0.4	N.D.	1.0	Trace

Disaccharide compositions (%) of untreated and fractionated CS were determined by HPLC on an amino-bound silica column after chondroitinase ABC digestion as described previously. ΔDi-0S, $\Delta^{4,5}$HexA(α1-3)GalNAc; ΔDi-4S, $\Delta^{4,5}$HexA(α1-3)GalNAc(4-O-sulfate); ΔDi-6S, $\Delta^{4,5}$HexA(α1-3)GalNAc(6-O-sulfate); ΔDi-diSE, $\Delta^{4,5}$HexA(α1-3)GalNAc(4,6-O-disulfate); ΔDi-diSB, $\Delta^{4,5}$HexA(2-O-sulfate) (α1-3)GalNAc(4-O-sulfate); ΔDi-diSD, $\Delta^{4,5}$HexA(2-O-sulfate)(α1-3)GalNAc(4-O-sulfate); ΔDi-tirS, $\Delta^{4,5}$HexA(2-O-sulfate)(α1-3)GalNAc(4,6-O-disulfate); N.D.: not determined.

TABLE 3: Kinetic parameters for the interaction of fractionated chondroitin sulfate with immobilized L-selectin.

	Fraction	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_A (M^{-1})	K_D (μM)
	CS (untreated)	6.16×10^2	7.52×10^{-3}	8.18×10^4	12.2
Whale cartilage	B (21 kDa)	7.58×10^3	2.27×10^{-2}	3.34×10^5	3.0
	C (15 kDa)	2.31×10^3	2.05×10^{-2}	1.13×10^5	8.8
	E (9 kDa)	6.05×10^2	3.42×10^{-2}	1.77×10^4	56.4
	CS (untreated)	3.32×10^2	1.30×10^{-2}	2.56×10^4	39.1
Bovine tracheal cartilage	C (21 kDa)	7.97×10^2	3.48×10^{-3}	2.23×10^5	4.5
	E (15 kDa)	1.22×10^2	2.30×10^{-3}	5.29×10^4	18.9
	G (9 kDa)	4.52×10^2	2.37×10^{-2}	2.00×10^4	49.9

The association rate constant (k_a), dissociation (k_d) rate constant, equilibrium association constant (K_A) and equilibrium dissociation constant (K_D) for the interaction of CS with immobilized L-selectin were determined by BIAcore. Standard errors of kinetic parameters obtained were within 15% by tripricate experiments.

The C-type lectin domain of L-selectin is known to interact with several glycan ligands directly. MAdCAM-1 [31], CD 34 [32], podocalyxin [33], and GlyCAM-1 [34] have been identified as L-selectin ligands which are expressed on cell surface of endothelial venule. Recently, it has been reported that penetration of white blood cell into an inflammatory tissue was inhibited by administration of anti-L-selectin antibody, and that penetration of white blood cell was suppressed significantly in L-selectin knock-out mouse [35, 36]. Consequently, it is assumed that L-selectin and the ligand play a role in penetration of white blood cell to nonlymphoid tissue, but the ligand is still unknown. Kawashima et al. revealed that L-selectin binding substance except for Sialyl-Lewis X exists in white matter and distal tubule which are nonlymphoid tissue and versican, a large chondroitin/dermatan sulfate (CS/DS) proteoglycan, interacts through its CS/DS chains with L- and P-selectin and CD 44 [15, 16, 18]. And we have reported that CS impacts intracellular transcription system through contact with L-selectin in OVA-sensitized mouse [12].

It has been shown that CSPG and CS are involved in anti-inflammatory reaction [37–39]. We also reported for the immunochemical reactivities of CS on murine splenocytes sensitized with OVA in order to demonstrate that CS could be associated with immune system. And we revealed that CS affects the pattern of cytokine production, differentiation of lymphocyte subsets, and level of IFN-γ mRNA in
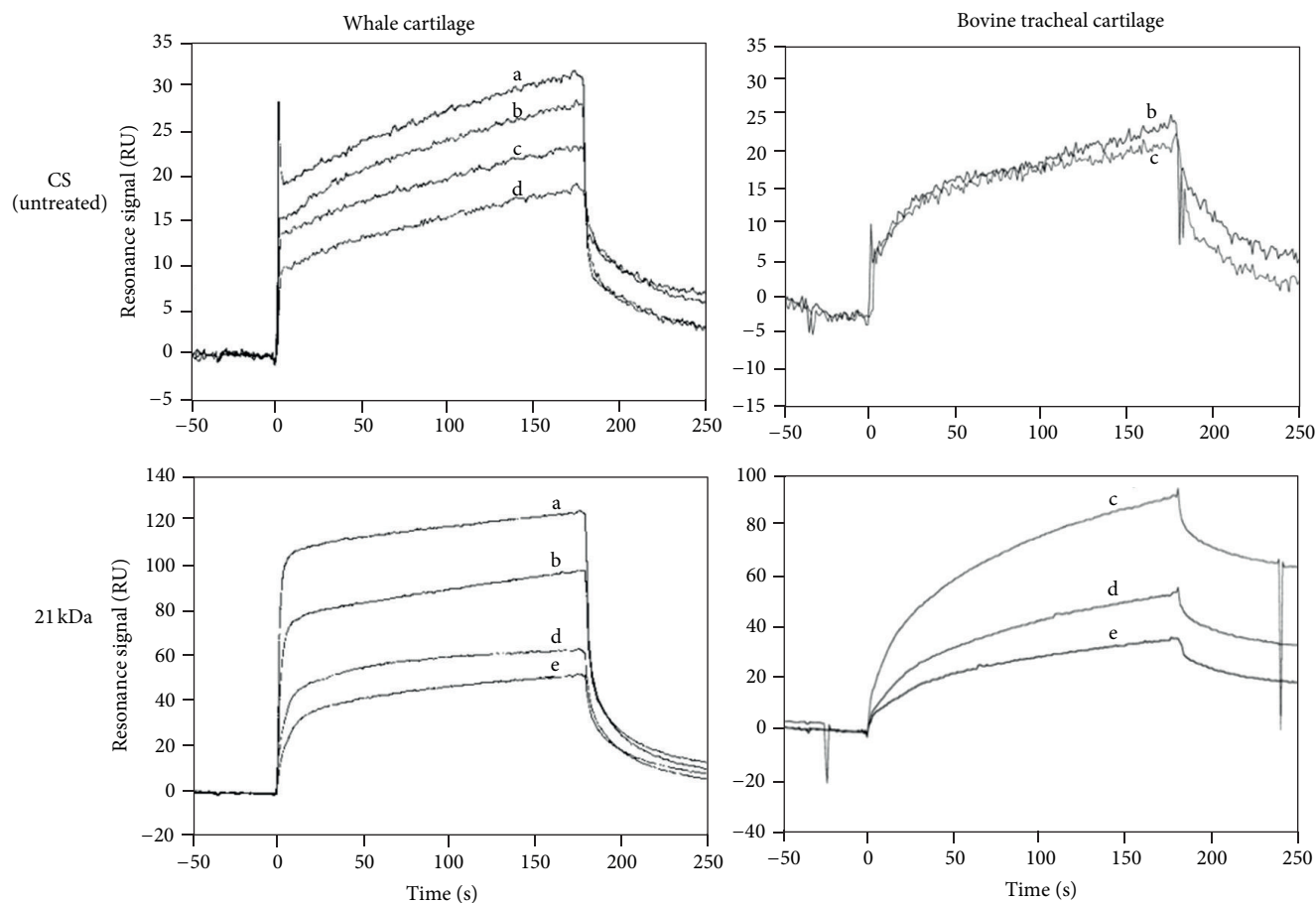
FIGURE 2: Sensorgrams of recording the interaction of fractionated chondroitin sulfate with immobilized L-selectin in the BIAcore. Various concentrations of original CS and fractionated CS (a, 50 μg/mL; b, 100 μg/mL; c, 250 μg/mL; d, 500 μg/mL; e, 1000 μg/mL) were continuously injected over sensor chip surfaces coupled to L-selectin at a 20 μL/min; after this, running buffer was injected and the response in resonance units was recorded as a function time.

the splenocytes of the mice immunized with OVA. Furthermore, it was shown that CS induced these reactions by interacting with L-selectin in sulfonation degree-dependent manner, and these findings suggest that CS could impact transcription system [12, 13]. Additionally, Zhou and his group have reported on the immune modulation by the animal model of experimental autoimmune encephalomyelitis (EAE) using CS and its disaccharide. It was shown that CS-A and disaccharide degradation of CSPG had an opposite effect on EAE [40]. Thus, it is suggested that CS has different immunochemical reactivity due to its structural variation. In this study, we focused on a variability of MW of CS in order to investigate the immunochemical reactivities of CS in detail and investigated the effect of CS with different MW on systemic immune systems.

5. Conclusion

In the past, many studies on the bioactivities of CS have been focused on the degree of its sulfonation patterns and they revealed that effects of CS on bioactivities differ with

the degree and positions of sulfonation CS chains. However, we clarified here that the immunoreactivity of CS is affected by the MW, length of leaner polysaccharide chains, of CS. Furthermore, even though the MW of CS was similar, the effect on the production of IFN-γ and IL-5 by mouse splenocytes was more augmented by CS with a narrow range of MW compared to CS with a broad range of MW. Namely, it is suggested that the length of polysaccharide chains of CS has also the impact on the immunoreactivity.

Conflict of Interests

There is no ethical problem or conflict of interests with regard to this paper.
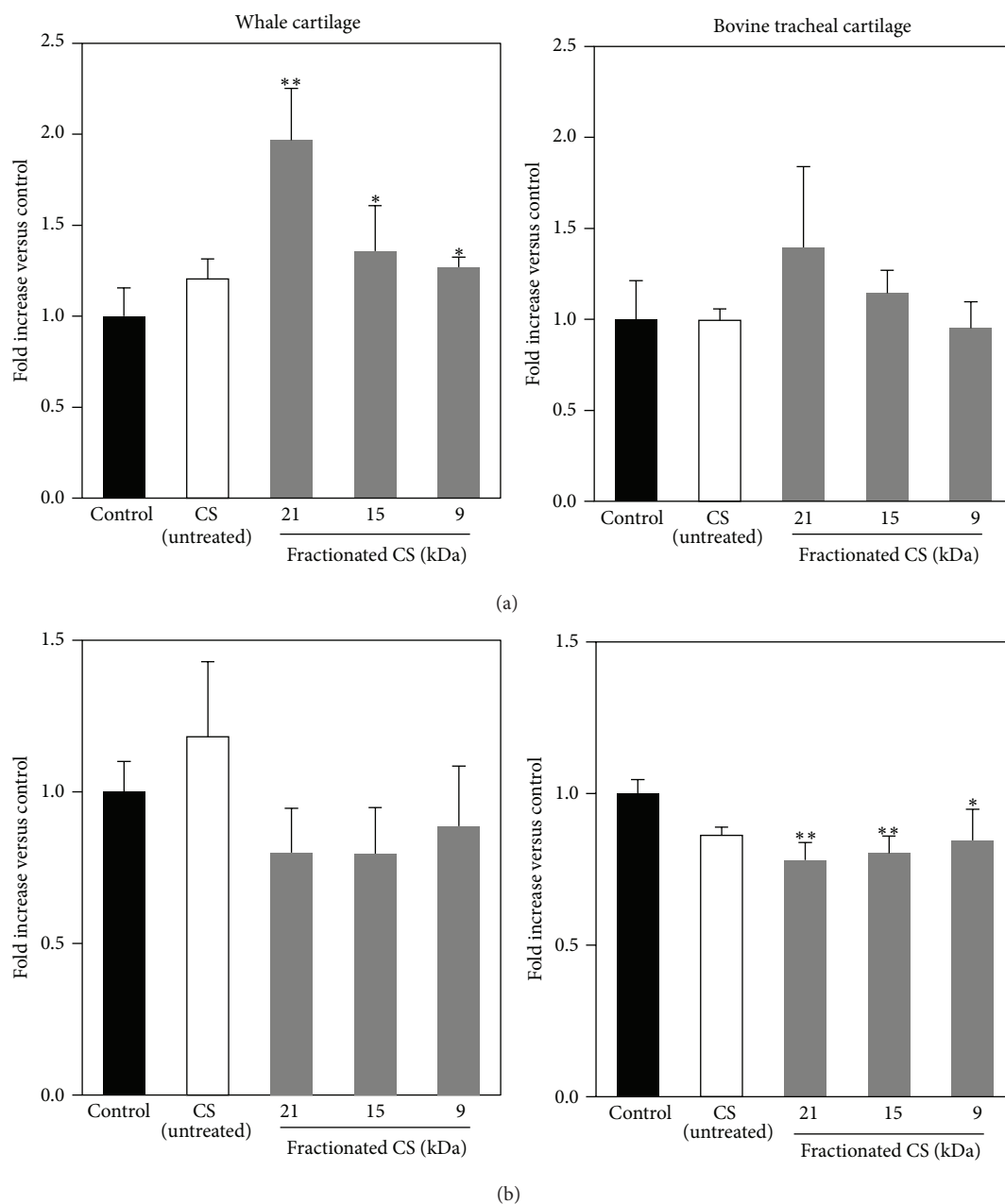
Acknowledgments

(a)

(b)

FIGURE 3: Effect of fractionated chondroitin sulfate on the cytokine productions of murine splenocytes *in vitro*. (a) IFN-γ production. (b) IL-5 production. BALB/c mice ($n = 5$) were intraperitoneally injected on day 0 and day 14 with 20 μg of OVA and 2 mg of Al (OH)$_3$ in a total volume of 400 μL. Splenocytes (5.0×10^6 cells/mL) were collected on day 15 and were cocultured with OVA (final concentration 100 μg/mL). The samples (each at a final concentration of 0.5 μM) or saline *in vitro* was added to the 24-well culture cluster in triplicate at 37°C in a CO$_2$ incubator for 48 hrs. The amounts of cytokines in the supernatant were measured by ELISA. $^*P < 0.05$, $^{**}P < 0.01$ (significantly different from control value). Error bars represent means ± S.D. for 6 wells.

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