# Potential Therapeutic Application of Chondroitin Sulfate/Dermatan Sulfate

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**Abstract:** Glycosaminoglycans (GAGs) are complex polysaccharides, which play important roles in cell growth, differentiation, morphogenesis, cell migration, and bacterial/viral infections. Major GAGs include heparin (Hep)/heparan sulfate, and chondroitin sulfate (CS)/dermatan sulfate (DS). Hep has been used for the treatment of thromboembolic disorders for more than 75 years, and has an established position in therapy today. CS/DS has attracted less attention and its clinical use is limited. However, CS/DS also have intriguing biological activities, which in turn should help in the development of CS/DS-based therapeutics. In this review, the following potential applications of CS/DS chains are discussed. (1) Sugar drugs for parasitic and viral infections. Particular CS variants appear to be involved in infections of various microbes, suggesting that CS/DS oligosaccharide sequences specifically interacting with microbes will lead to the development of inhibitory drugs for these infections. (2) Regenerative medicine. Biological activities of CS/DS chains possibly involve various growth factors, also known as Hep-binding growth factors. Specific CS/DS chains recruit growth/neurotrophic factors and/or potentiate their activities, suggesting that minute amounts of functional CS/DS chains can be utilized for tissue regeneration instead of signaling proteins. (3) Anti-tumor drugs. Specific saccharide structures in CS/DS chains appear to be involved in tumor cell proliferation and metastasis. The detection and identification of such CS/DS saccharide sequences would be an important contribution to cancer therapy.

**Key Words:** Glycosaminoglycan, chondroitin sulfate, dermatan sulfate, virus, infection, tissue regeneration, neurite outgrowth, osteoarthritis, cancer.

## INTRODUCTION

Glycosaminoglycans (GAGs) are linear polymers composed of alternating amino sugar and hexuronic acid residues and distributed as side chains of proteoglycans (PGs) in the extracellular matrix (ECM) or at the cell surface of animal tissues. Major GAGs include chondroitin sulfate/dematan sulfate (CS/DS) and heparan sulfate/heparin (HS/Hep). Although the polysaccharide backbones of these GAGs are simple, repetitive linear chains, these structures acquire a considerable degree of variability by extensive modifications involving sulfation and uronate epimerization [1], which are the basis for the wide variety of domain structures with biological activities [2].

Although critical roles of HS/Hep in developmental processes and specific signaling pathways have been demonstrated [3], CS/DS chains have been considered to participate only in structural stabilization and attracted less attention until recently. However, accumulating evidence implies important biological functions of CS/DS chains in cytokinesis, cell proliferation, differentiation, migration, tissue morphogenesis, organogenesis, infection, and wound repair [4-7]. CS/DS interact with a wide variety of key molecules, such as growth factors, cytokines, chemokines, and adhesion molecules, via specific saccharide domains within the chains (Fig. (**1**), Table **1**). As shown in Table **1**, CD/DS-binding proteins include various Hep-binding growth factors. Because of their sulfation structure, most Hep-binding growth factors are presumed to interact with CS/DS. The biological significance of such interactions is emerging.

CS/DS chains are composed of alternating units of *N*-acetyl-D-galactosamine (GalNAc) and either glucuronic acid (GlcA) or iduronic acid (IdoA), and the sugar backbones can be sulfated mainly at the C2 position of uronic acid residues and at the C4 and/or C6 positions of GalNAc residues, forming various disaccharide units as shown in Fig. (**2**) [5]. Diverse modifications through C5 epimerase and *O*-sulfotransferases are responsible for the structural heterogeneity of the CS/DS chains. The particular functional domain structures, which are formed by combinations of these various disaccharide units, may participate in specific binding to bioactive molecules, and hence may greatly influence the overall functions of CS/DS.

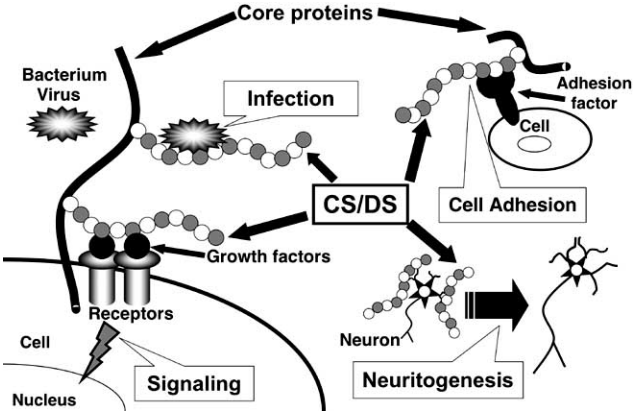


**Fig. (1).** Schematic drawing of the biological functions of CS/DS on the cell surface or in the extracellular matrix. CS/DS chains play important roles on the cell surface or in the extracellular matrix. For instance, they regulate growth factor signaling, promote neurite outgrowth, are involved in cell adhesion by interacting with adhesion factors, and are recognized by microorganisms as infection receptors. In the figure, CS/DS chains are represented by alternating open and closed circles.

Critical roles of CS/DS in various physiological events have recently been demonstrated using model organisms such as *Caenorabditis elegans*, *Drosophila melanogaster*, zebrafish, and mice. When *C. elegans* is treated with chondroitin synthase dsRNA, most oocytes and fertilized eggs die *in utero*. Analysis by four-dimensional microscopy revealed that fertilized eggs laid within 11-12 hours of RNAi treatment could not complete cell division, particularly cytokinesis [48]. In the zebrafish embryo, CS/DS chains are present at the interface of the somites and the notochord where spinal motor axons extend ventrally to establish the midsegmental ventral motor nerves. Injection of a CS-degrading enzyme prior to motor axon outgrowth effectively removed all CS/DS chains and induced abnormal axonal outgrowth in many of the ventral motor nerves [49]. This suggests that CS chains normally constrain the outgrowth of the ventral motor nerves. Mice deficient in the chondroitin-4-sulfotransferase 1 (*C4st1*)

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**Table 1. CS/DS-Binding Proteins**

| CS/DS-Binding Proteins | Refs. | Related Diseases or Functions |
|---|---|---|
| **ECM Components** | | |
| Type II collagen | [8] | primary osteoarthritis, spondyloepiphyseal dysplasia congenita etc. |
| Type V collagen | [8, 9] | Ehlers-Danlos syndrome, tuberous sclerosis |
| Type VII collagen | [8] | epidermolysis bullosa |
| Tenascin-X | [10] | Ehlers-Danlos syndrome |
| Opticin | [11] | binding to growth hormone |
| **Coagulation factor** | | |
| Heparin cofactor II | [12, 13] | congenital coagulation disorders |
| **Growth Factors** | | |
| Fibroblast growth factor (FGF)-2, -10, -16, -18 | [14-18] | wound healing, angiogenesis-related diseases (cancer etc.) |
| Keratinocyte growth factor (KGF) (FGF-7) | [13, 19, 20] | tissue repair, cancer |
| Heparin-binding EGF-like growth factor (HB-EGF) | [14-16] | arteriosclerosis, liver cancer, wound healing |
| Hepatocyte growth factor/scatter factor (HGF/SF) | [13, 20, 21] | liver diseases (fulminant hepatitis etc.), myocardial infarction, breast cancer, non-small cell lung cancer |
| Midkine (MK) | [16, 17, 22] | cancer |
| Pleiotrophin (PTN, HB-GAM) | [16, 17, 23, 24] | cancer |
| Platelet derived growth factor (PDGF) | [25] | arteriosclerosis, malignant tumor |
| Vascular endothelial growth factor (VEGF) | [15, 20] | diabetic retinopathy, rheumatoid arthritis, hyperthyroidism, solid tumor |
| Transforming growth factor-β (TGF-β) | [26] | cancer, liver fibrosis, cirrhosis, pulmonary fibrosis, glomerulonephritis, diabetic nephropathy, scleroderma etc. |
| Glial-derived neurotrophic factor (GDNF) | [15, 18] | extensive aganglionosis (Hirschsprung's disease) |
| Brain-derived neurotrophic factor (BDNF) | [15, 18] | autism |
| Heparin affin regulatory peptide (HARP) | [27] | maturation of nerve cells, tumor growth |
| **Virus protein** | | |
| Glycoprotein C | [28, 29] | herpes simplex virus infection |
| **Cell Adhesion Molecules** | | |
| CD-44 | [30] | inflammation, malignant tumor |
| L-Selectin | [30] | leukocyte adhesion deficiency |
| P-Selectin | [30] | inflammation, thrombosis |
| RANTES | [13, 31, 32] | allergic inflammation |
| von Willebrand factor | [33] | von Willebrand disease |
| **Chemokines** | | |
| Interferon-γ (IFN-γ) | [34] | antiviral and antitumor actions |
| Interleukin-8 (IL-8) | [31, 35] | arthritis, acute nephritis, sepsis, ischemia-reperfusion injury in brain etc. |
| Macrophage inflammatory peptides 1α (MIP-1α) | [31] | myeloma bone disease |
| Monocyte chemoattractant protein-1 (MCP-1) | [31] | atherosclerosis, multiple sclerosis |
| Secondary lymphoid tissue chemokine (SLC) | [30] | inflammation |
| γ-Interferon inducible protein-10 (IP-10) | [30] | inflammation, cancer |
| Stromal cell-derived factor-1β (SDF-1β) | [30] | hematopoiesis |
| Platelet factor 4 (PF4) | [30, 36] | hematopoiesis, angiogenesis |
| **Proteases** | | |
| Leukocyte elastase | [37] | lung emphysema, clotting disorders |
| Matrix metalloproteinase-2 | [38] | tumor cell invasion and metastasis |
| **Others** | | |
| β-Amyloid peptide (Aβ) | [39] | Alzheimer disease |
| Cardiotoxins from spitting cobra venom | [40] | corneal opacity, blindness |
| α-Defensin | [41] | antimicrobial peptide |
| EGF-TM7 receptors CD97 and EMR2 | [42, 43] | cell adhesion, cell migration |
| Extracellular superoxide dismutase (EC-SOD) | [44] | oxidant-mediated diseases |
| Lipoprotein lipase (LPL) | [45] | hyperlipoproteinemia type I |
| Tumor necrosis factor-alpha-stimulated gene 6 (TSG-6) | [46] | inflammation, autoimmune disorders |
| Thyroglobulin | [47] | Graves' disease, Hashimoto's thyroiditis |

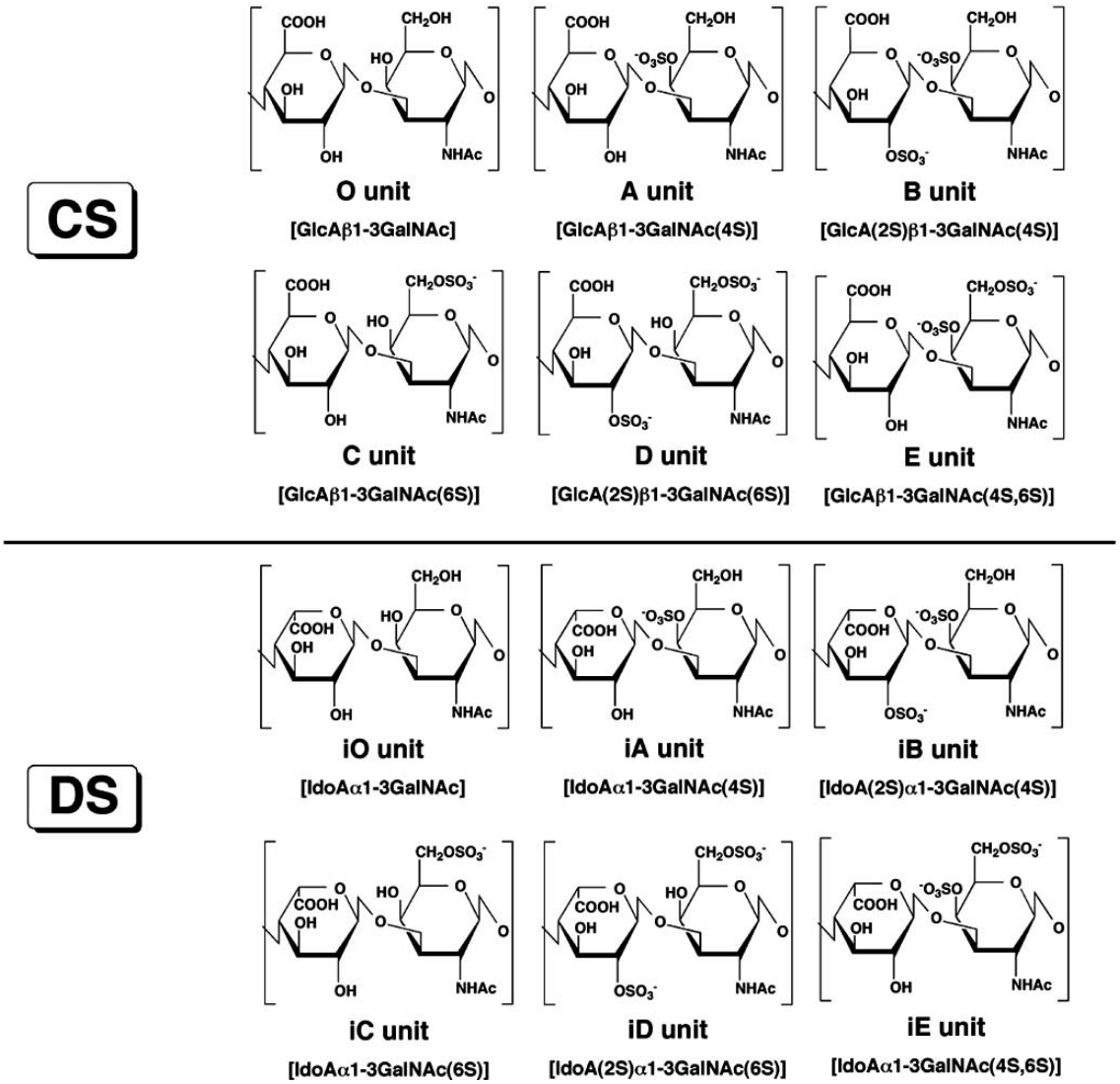


**Fig. (2).** Typical disaccharide units found in CS/DS chains. The structure and designation of disaccharide units predominantly present in CS/DS chains are given. "i" in the DS disaccharide units represents IdoA. 2S, 4S, or 6S stands for 2-*O*-, 4-*O*-, or 6-*O*-sultate, respectively.

gene, which encodes an enzyme specific for the transfer of sulfate groups to the C4 position of GalNAc residues of CS chains, were generated by a gene trap method. The deficiency causes severe chondrodysplasia characterized by a disorganized cartilage growth plate as well as specific alterations in the orientation of chondrocyte columns. This phenotype is associated with a CS imbalance, mislocalization of CS in the growth plate, and an imbalance of apoptotic signals. These results show that chondroitin 4-*O*-sulfation by C4st1 is required for the proper localization of CS, modulation of distinct signaling pathways, and cartilage growth plate morphogenesis, indicating an important biological role of the different CS chains in mammalian development [50]. A disturbance of GAG sulfation can produce a human genetic disorder. Thiele *et al.* [51] studied a distinct form of spondyloepiphyseal dysplasia (SED Omani type). By using a genome-wide linkage approach, a missense mutation in the chondroitin 6-*O*-sulfotransferase (*C6ST-1*) gene was identified. The recombinant C6ST-1 with the missense mutation loses all activity. A disaccharide compositional analysis of CS chains of patients showed marked undersulfation of CS, in particular, a reduction in 6-*O*-sulfated disaccharide. Thus, the mutation in *C6ST-1* causes a specific but generalized defect of CS chain sulfation resulting in chondrodysplasia with major involvement of the spine.

In this review, we describe the biological functions of CS/DS under both physiological and pathological conditions, and discuss the potential applications of CS/DS and their derivatives to therapeutics. CS/DS are good research seeds for developing medicines.

## I. SUGAR DRUGS FOR PARASITIC AND VIRAL INFECTIONS

Many bacteria, parasites, and viruses exploit cell surface GAGs as adhesion receptors (Table **2**). Of the several GAG types present in animal cells, HS is the beststudied, associating with pathogens including dengue virus, herpes simplex virus type 1 (HSV-1), human herpesvirus (HHV) 8, human papillomavirus, hepatitis C virus, and *Neisseria gonorrhoeae* [78, 82-84]. In contrast, reports of the involvement of CS/DS in the adhesion of pathogens to cells are limited. The Lyme disease spirochete, the malarial parasite, and HSV-1 utilize cell surface CS/DS chains as receptors [28, 54, 85, 86].

Most interactions between adherent microorganisms and cell surface GAGs may be nonspecific but ionic because of a high charge density of GAGs due to a clustering of sulfate groups. However, in some cases unique sugar sequences in GAG chains appear to be involved in microbial adherence [87]. It is critical to identify the oligosaccharide sequences in the GAG chains, which specifically interact with microbial adherent proteins. A comprehensive understanding of the structural interactions involved in microbial adherence to the cell surface GAGs would be valuable not only in the elucidation of the

**Table 2. Human Pathogenic Microorganisms which Recognize GAGs as Cell Surface Receptors**

| Microbes | Refs. | Related Diseases |
|---|---|---|
| **Bacteria** | | |
| *Bordetella pertussis* | [52, 53] | pertussis (whooping cough) |
| *Borrelia burgdorfei* | [54] | Lyme disease |
| *Chlamydia trachomatis* | [55, 56] | genital chlamydia, endemic trachoma, etc. |
| *Haemophilus influenzae* | [57] | meningitis, pneumonia |
| *Helicobacter pylori* | [58] | gastritis, stomach ulcer, duodenum ulcer, stomach cancer |
| *Neisseria gonorrhoeae* | [59] | gonorrhea |
| *Staphylococcus aureus* | [60] | toxic shock syndrome, suppuration |
| *Streptococcus gordonii* | [61] | infectious endocarditis |
| *Streptococcus mutans* | [62] | dental caries |
| **Parasites** | | |
| *Plasmodium falciparum* | [63, 64] | malaria |
| *Leishmania amazonensis* | [65] | cutaneous leishmaniasis |
| *Leishmania donovani* | [66] | visceral leishmaniasis |
| *Trypanosoma cruzi* | [67] | Chagas' disease |
| **Viruses** | | |
| Herpes simplex virus (HSV) -1, -2 | [29, 68] | perioral and genital lesions and encephalitis |
| Varicella zoster virus | [69] | chickenpox and shingles |
| Cytomegalovirus | [70] | Kaposi's sarcoma |
| Epstein-Barr virus | [71] | Burkitt's lymphoma |
| Human herpes virus 8 | [72] | Kaposi's sarcoma, Castleman's disease |
| Adenovirus | [73] | acute respiratory infections |
| Dengue virus | [74] | dengue hemorrhagic fever, dengue shock syndrome |
| Hepatitis B virus (HBV) | [75] | hepatitis |
| Hepatitis C virus (HCV) | [76] | liver cancer and cirrhosis |
| Human immunodeficiency virus (HIV) | [77] | acquired immunodeficiency syndrome (AIDS) |
| Human papillomavirus | [78] | cervical cancer and genital warts |
| Respiratory syncytial virus | [79, 80] | respiratory infection, particularly among infants and young children |
| Vaccinia virus | [81] | smallpox |

mechanism of microbial infection but also in the design of GAG oligosaccharides or mimetic therapeutics for the treatment of related diseases.

GAG-binding microorganisms are summarized in Table **2**. Most of them have not been tested for whether they interact with CS. Only low sulfated CS-A or CS-C polysaccharides have been used for experiments. Highly sulfated CS variants, such as CS-E, have not been investigated, except for HSV [28, 29, 86]. Therefore, it is worth examining whether some of the microorganisms in Table **2** interact with various CS preparations including highly sulfated CS variants and recognize cell surface CS/DS as a receptor.

Here we review the interaction of GAGs with microbes, which have been demonstrated to utilize cell surface CS/DS as captors for infection, and the application of CS/DS and their derivatives as inhibitory drugs.

### (1) Herpes Simplex Virus Type 1

Eight herpes viruses, namely HSV-1, HSV-2, varicella-zoster virus, human cytomegalovirus 1, Epstein-Barr virus, HHV-6, HHV-7, and HHV-8, or Kaposi sarcoma–associated herpes virus infect humans [87]. HSV-1 is a neurotropic cytolytic virus that can establish asymptomatic latent infections in neurons of the peripheral nervous system. The most common form of the disease caused by HSV in humans manifests as mucocutaneous lesions, which occur usually in or near the mouth, on the cornea, or on genital tissues. Less frequently, HSV can also cause a life-threatening disease affecting vital organs, including encephalitis in apparently normal adults and disseminated disease in infants and immunocompromised individuals.

HSV-1 enters cells by inducing fusion between the viral envelope and the cell membrane. Cell surface HS is the initial receptor for the binding of HSV-1 to cells [68]. Cells treated with heparinases or altered by mutations that prevent HS biosynthesis, have a reduced capacity to bind the virus, but are partially resistant to infection [68, 88]. HSV-1 was shown to infect polarized MDCK cells which express CS predominantly on their apical surfaces [89]. It was found that HSV-1 bound to and infected mutant gro2C cells exposing only CS on the cell surface, which were derived from mouse fibroblast L cells and a variant deficient in HS expression, and that the cell line designated sog9 lacking both CS and HS expression is resistant to HSV-1 [85, 90, 91]. These results indicate that cell surface CS is also a receptor for HSV-1. Although CS is regarded as an auxiliary receptor that in the absence of HS chains can support the initial interaction of HSV-1 with cells, a major CS-binding domain of HSV-1 attachment glycoprotein C (gC) does not completely overlap with the HS-binding site [28]. This suggests that cell surface CS chains are also specifically recognized by HSV-1 and function as adhesion receptors even in the presence of HS.

Recently, Bergefall *et al.* [29] have demonstrated that CS chains with a high content of the oversulfated E [GlcAβ1-3GalNAc(4,6-*O*-disulfate)] units (Fig. (**2**)) potently inhibit HSV-1 infectivity and di-

**Table 3. Oligosaccharides from Functional Domains of CS/DS**

| Oligosaccharides[a] | Refs. |
|---|---|
| DS hexasaccharide binding to heparin cofactor II with high affinity: iB-iB-iB[b] | [12] |
| CS dodecasaccharide binding to *P. falciparum*-infected red blood cells: GlcA-GalNAc-GlcA-GalNAc-GlcA-GalNAc-GlcA-GalNAc-GlcA-GalNAc-GlcA-GalNAc + two or three 4-*O*-sulfate groups | [93] |
| CS-E tetrasaccharide binding to selectins and chemokines: E-E | [30] |
| CS-E octasaccharide binding to type V collagen: E-E-E-E | [94] |
| Chemically synthesized CS tetrasaccharide stimulating the outgrowth of neurites: E-E[c] | [95] |
| CS-E octasaccharide interacting with gC of HSV-1: octasaccharides with a minimum of two consecutive E units (-E-E-)[d] C-E-E-C, E-E-A-C, E-E-A-A, E-E-E-C, E-E-E-A, and E-E-E-E | [29] |
| PTN-binding octasaccharies from embryonic pig brain CS/DS: ΔC-C-D-C[e], ΔA-C-D-C[e], ΔC-A-D-C[e], ΔD-C-D-C[e], ΔC-D-(i)D-C[e,f], and ΔE-D-(i)A-D[e,g] | [96] |
| CS-C hexasaccharides recognized by an antibody WF6: ΔD-C-C-C[e] and ΔC-C-A-D[e] | [97] |

[a]Abbreviations used for the disaccharide units, see Fig. (**2**).
[b]The reducing terminal sugar residue of this hexasaccharide is converted from GalNAc to 2,5-anhydrotalitol.
[c]The reducing terminal GalNAc residue is masked with an allyl group.
[d]S. Naito, S. Yamada, and K. Sugahara, unpublished data.
[e]The nonreducing terminal sugar residue is an unsaturated hexuronic acid.
[f]The disaccharide unit penultimate from the reducing end is D or iD.
[g]The disaccharide unit penultimate from the reducing end is A or iA.

rectly interact with gC of HSV-1, indicating that E units in CS chains play a critical role in the recognition by HSV-1 gC. The minimum size of a CS-E oligosaccharide required for inhibition of the binding between immobilized CS-E and gC was demonstrated to be an octasaccharide. To further characterize the binding of CS-E to gC, six structurally defined octasaccharide fractions prepared from CS-E were used in sugar microarray experiments [13, 92], in which the lipid-derivatives of CS-E oligosaccharide fractions were immobilized on a nitrocellulose membrane through the hydrophobicity of the lipid moiety and their binding to gC was detected by using an anti-gC antibody. It was found that all of the octasaccharide fractions bound to gC (S. Naito, S. Yamada and K. Sugahara, unpublished results). The structure of the major octasaccharide in each fraction is summarized in Table **3**. The observed preference suggests that gC can recognize octasaccharides with at least two consecutive E units. Identification of the specific oligosaccharide sequences, which can inhibit HSV-1 infections, will form a structural basis for the development of inhibitors of HSV-1 infections in humans.

### (2) Malarial Parasite

Malaria is a deadly infectious disease in the tropical and subtropical regions of the world. Despite the availability of various drugs for treatment, malaria continues to be a leading cause of deaths with 2-3 million people dying from it annually [98]. In areas where malaria is endemic, women are highly susceptible to *Plasmodium falciparum* malaria during pregnancy, especially if the pregnancy is their first [99, 100]. Pregnancy-associated malarial complications have been recognized for more than 100 years [100]. Nevertheless, until recently, the underlying mechanism for the adherence of infected red blood cells in the placenta was unknown. The reason for the susceptibility of pregnant woman is that the development of the placenta provides a new receptor for *P. falciparum* that is not expressed on the vascular endotherial surface. Several studies have established that *P. falciparum*-infected red blood cells specifically bind to low sulfated CS-A, which is located in the intervillos space of the placenta [100]. In this section, the role of CS in the adherence of *P. falciparum*-infected red blood cells in the human placenta, which leads to pregnancy-associated malaria, is described.

Rogerson *et al.* [101] discovered that a population of *P. falciparum* strains can adhere to CHO cells and the binding is mediated by CS-A. The infected red blood cells were unable to adhere to CHO mutant cells deficient in the expression of CS [101]. Binding of the infected red blood cells to CHO cells was inhibited by CS-A but not by CS-C, DS, HS, or Hep. The structural requirement for the adhesion of the infected red blood cells to the CS chains of the placental CS-PG receptor has been determined by an inhibition-based analysis using CS with various sulfation patterns [93]. It was inferred that the adhesion of the infected red blood cells involves the participation of both A [GlcAβ1-3GalNAc(4-*O*-sulfate)] and O [GlcAβ1-3GalNAc] disaccharide units (Fig. (**2**)) of the CS chains. CS-A chains having 30-52% A units maximally inhibited the binding of the infected red blood cells to placental CS-PG, whereas CS-A chains containing 3-11% or >80% A units were less inhibitory [93]. It appears that a specific distribution pattern of the sulfate groups is involved in the binding of the infected red blood cells.

The minimum size, required for maximum inhibition of the adherence of the infected red blood cells to the placental CS-PG, was demonstrated to be a dodecasaccharide [93]. Based on these findings, dodecasaccharides composed of two A units and four O units or three A units and three O units appear to play a critical role in the recognition by *P. falciparum*-infected red blood cells (Table **3**). Since the adherence of the infected red blood cells to CS-A is an effective target for the development of therapeutics and/or a vaccine, identification of the specific dodecasaccharide sequence composed of A and O units is needed.

## II. REGENERATIVE MEDICINE

CS/DS-PGs play important roles in the morphogenesis and maintenance of various tissues through interactions with cell adhesion molecules, ECM molecules, and growth factors. In this section, studies on the functions of CS/DS-PGs in tissue regeneration are summarized.

### (1) Repair of the Central Nervous System

In the central nervous system (CNS), regenerative capacity is limited in adulthood. However, recent studies have illuminated the repair mechanisms (regeneration and neurogenesis). The CNS is a rich and complex source of proteins modified with CS/DS chains. During the brain's development, levels of CS/DS-PGs are particularly high and finely tuned expression patterns suggest important functions at all stages of development [6, 102, 103]. CS/DS-PGs can also function in the CNS under pathological conditions. In Alzheimer's disease, CS and DS localize to the characteristic lesions [104] and were shown to be potent enhancers of amyloid fibrillogenesis [105]. In epilepsy, alterations in the occurrence of CS-PG have been observed [106].

CS/DS-PG levels are elevated under various pathological conditions both in the CNS and in the periphery, where these compounds are associated mainly with wound repair processes [6, 107, 108]. After injury to the CNS, glial scar tissue forms and expresses multiple axonal growth inhibitory molecules to present a physical and molecular barrier to axonal regeneration [109]. CS/DS-PG molecules are present in the glial scar and generally inhibitory to CNS repair [109]. CS/DS-PGs form a homogeneous meshwork around the wound and seal the injury site to prevent the diffusion of mediators of damage to the surrounding healthy tissue. Since removal or reduction of the CS/DS chains in the lesion zone by enzymatic digestion promotes the regeneration of neurites in the spinal cord [110], CS/DS moieties of CS/DS-PGs are considered to be necessary parts of the inhibitory structures.

CS/DS promote the outgrowth of neurites in embryonic rat brain neurons [111]. CS-E and oversulfated DS also promote the outgrowth of neurites in hippocampal neurons [112]. Oversulfated CS disaccharides as well as human brain-derived appican PG have been implicated in the support of neurite outgrowth [113]. CS-PGs promote the survival of neurons [114, 115], in which the Rho signaling pathway plays an important role [116]. Based on these findings, CS/DS-PGs have a very complicated role in CNS repair. CS/DS-PGs seem to either promote or inhibit neurite outgrowth depending on neuronal cell types, experimental conditions, and the fine structure of the CS/DS side chains.

CS/DS themselves appear to modulate neuritogenesis either positively or negatively. The heterogeneity of CS/DS structures may make CS/DS-PGs versatile and able to act both as a barrier and as regulators of bioactive proteins. CS/DS-PGs interact with many different soluble and membrane-associated molecules [117]. Binding partners of CS/DS include growth factors and neurotropic factors as well as structural components of cells or the ECM (Table **1**).

A variety of Hep-binding growth factors and cytokines are involved in biological processes in the CNS including stem cell proliferation, neurogenesis, gliogenesis, and neural network formation through interaction with GAGs at the cell surface and in the ECM [102, 117]. Interaction of pleiotrophin (PTN) with CS/DS chains of receptor-type protein tyrosine phosphatase ζ (PTPζ) on neuronal cells induces dimerization of PTPζ, which turns on the downstream intracellular signaling events leading to neuritogenesis [23]. The particular CS/DS preparation from the telencephalon of embryonic day-14-old rats, whose structure is characterized by iA, D, iD, E, and/or iE units (see Fig. (2)), possesses the potential to promote the proliferation of neural stem/progenitor cells mediated by fibroblast growth factor (FGF)-2 [118]. Oversulfated CS/DS motifs have been isolated from developing brains which are able to interact with a variety of different cytokines with high affinity and may modulate the activity of these cytokines [17, 96, 119, 120]. Angiogenesis is another factor crucial to efficient CNS repair. CS/DS-PGs may also play a role in promoting angiogenesis together with some growth factors [121, 122]. These observations suggest that CS/DS-PGs do not impose a mechanical barrier to regeneration but rather regulate the proliferation and differentiation of the neuronal cells through CS/DS-binding molecules.

The sulfation pattern of CS/DS chains directly influences the binding to bioactive proteins and regulates the function of CS/DS-PGs in CNS repair. A morphometric analysis of primary neurons grown on CS/DS substrata revealed a good correlation between their characteristic sulfation patterns and neuritogenic properties. The neurons cultured on a substratum coated with a CS-D or DS preparation from ascidians, which contains a larger proportion of the characteristic D or iD unit, respectively, exhibited a flattened cell soma with dendrite-like multiple neurites [112]. On the other hand, the neuons grown on a substratum coated with a CS-E or DS preparation from hagfish notochord, which is characterized by a predominance of the E or iE unit, respectively, showed a round cell soma and a smaller number of prominent long neurites [112]. These findings suggest that neuritogenic activities of the oversulfated CS/DS chains are dependent on the sulfation patterns rather than the simple charge densities of the respective GAG chains. Thus, the profile of sulfation of CS/DS chains is a crucial factor regulating the proliferation of neural stem cells in the CNS through interaction with growth factors. Although the specific sulfation patterns required for the activities are yet unknown, a series of PTN-binding octasaccharides were isolated and characterized using a PTN-immobilized column after partial fragmentation of CS/DS chains from embryonic pig brain followed by labeling with a fluorophore [96], which facilitated the isolation and sequencing of minute amounts of the oligosaccharides at a low picomole level [123, 124].

The neurogenerative functions of CS/DS may be associated with the precisely sulfated distinct structures embedded in the CS/DS chains, and are exerted mainly through specific interactions with bioactive protein factors such as growth factors and neurotrophic factors [102, 117]. Thus, CS/DS polysaccharides or oligosaccharides containing such active sequences bear great therapeutic potential. The application of CS-PG promotes the survival of fetal and neonatal murine retinal ganglion cells in retinae explanted to the chorioallantoic membrane of the chick [125]. Recent studies demonstrated that the degradation products of CS-PGs were highly effective in promoting axonal growth, enhancing microglial activation, and controlling T-cell functions [126]. Furthermore, sulfated monosaccharides and disaccharides from CS/DS were used to compete with the intact CS and Hep for binding amyloid-beta, indicating that they may be effective inhibitors of the GAG-induced amyloid formation [127]. The interesting effects observed after the alteration of CS/DS-PG function with a specific CS/DS-degrading enzyme in models of CNS damage [110] have also stimulated pharmacological interest in these molecules. CS/DS polysaccharides from various sources, their degradation products, CS/DS-related oligosaccharides, and specific CS/DS-degrading enzymes are expected to have pharmacological applications in the future. Analyses using these molecules and enzymes may also lead to a better understanding of the endogenous repair mechanisms in the CNS.

### (2) Liver Regeneration

Highly sulfated CS/DS structures also appear to be involved in liver development. It has been demonstrated by affinity chromatography that hepatocyte growth factor/scatter factor (HGF/SF) binds strongly to DS [128]. Heparin-binding EGF-like growth factor (HB-EGF), which is a key factor for hepatocyte progression during liver regeneration [129], interacts with highly sulfated CS/DS [14, 20]. During liver regeneration, transient changes in the structure of GAGs in the liver are associated with hepatocyte proliferation [130]. In the fibrous lesions of livers with cirrhosis, a remarkable increase in

CS/DS content as well as a decrease in the degree of sulfation of the CS/DS chains and the proportion of IdoA content in the CS/DS chains have been observed, when compared with those in nonfibrous lesions [131]. A deficiency in xylosyltransferase 2, which is an enzyme involved in GAG biosynthesis [3], causes a loss of a majority of GAGs in the liver and results in significant hepatic abnormalities including biliary tract hyperplasia, liver fibrosis, and biliary cysts [132]. These findings indicate that liver GAGs are influential in hepatocellular proliferation and differentiation. Thus, exogenous administration of the functional domains of CS/DS, which specifically interact with the factors for liver regeneration such as HGF and HB-EGF (Table **1**), may regulate the physiology of hepatic functions. CS/DS chains and their derivatives are potential candidates for development of therapeutics for liver regeneration.

**(3) Management of Osteoarthritis**

Osteoarthritis (OA) is the most common form of arthritis, affecting knees, fingers, and hips usually among the elderly. The first attempts at improving the structure and function of the connective tissues of synovial joints and thereby alleviating the symptoms of OA, were based on vague assumptions that the administration of precursors of ECM components would help articular cartilage cells to replace the lost environment [133]. It was presumed that CS would replace hyaluronic acid (HA) as a lubricant and reduce levels of fibrinogen in inflamed joints and that this would have a therapeutical advantage [134]. The possibility of positively interfering with the *in vitro* connective tissue cell repair process using sulfated polysaccharides was first described in the mid 1970s [135, 136], and CS has been used as a symptomatic slow-acting drug for OA [137].

Although biological activity of orally delivered CS capable of modifying the structure of connective tissues in a positive way (the structure-modifying effects) was reported in the treatment of OA, polysaccharides such as CS are poorly absorbed through the digestive system [138, 139]. It has been demonstrated that the half-life of CS in the circulatory system is 3–15 min, based on the pharmacokinetic study of intravenously administered CS [140]. This indicates that orally administered CS is not systemically distributed to connective tissues such as cartilage and skin, and that exogenously administered CS may indirectly stimulate chondrocytes to synthesize ECM components. The mechanism of action of orally administered CS might be mediated by other systems.

A couple of randomized, double-blind, placebo controlled therapeutic trials have demonstrated that the introduction of oral CS is effective in alleviating the painful symptoms of OA [141, 142]. However, the mechanisms of action of CS against cartilage metabolism are not well understood. The therapeutic effects of CS may be related to its *in vitro* action. A number of *in vitro* studies have been performed to determine the mode of action of CS. Articular cartilage is a highly specialized tissue that contains only one type of cell, chondrocytes, and the metabolic activity of these cells is regulated by several mediators, such as cytokines, hormones, and growth factors, produced locally by the chondrocytes themselves and also by neighboring tissues [143]. CS might have some chondroprotective effects in OA disease as it is able to interact and modulate the functions of key cytokines and enzymes directly involved in the development and the progression of the OA disease.

Chondrocyte functions are influenced by ECM composition. CS has profound effects on the synthesis and turnover of the structural components of the ECM in human connective tissues [135, 136, 144], and may thus affect connective tissue repair. In various models of cartilage culture or of isolated chondrocytes, CS has demonstrated the capacity to stimulate the synthesis of PGs, aggrecans, and HA [145-149]. Physiological concentrations of CS were also able to significantly reduce the downstream effects of IL-1 and reduce the activities of collagenase, proteoglycanase, and matrix metalloproteases-1 and -3 [149, 150].

The possibility that CS protects against apoptosis has also been investigated. In cultures of human osteoarthritic chondrocytes, CS is able to prevent apoptosis and the degradation of cartilage can probably be explained on the basis of its capacity to reduce the nuclear translocation of the transcription factor NF-κB induced by IL-1β [151]. Also, in cultured equine chondrocytes, CS significantly reduced IL-1β-enhanced expression of inducible nitric oxide synthase (iNOS) that was paralleled by an increased release of nitric oxide (NO). CS downsizes nitrite concentrations in the supernatants of these IL-1β-stimulated cultures [152]. Recently Legendre *et al.* determined the effect of CS on IL-1β-induced expression of genes related to catabolic, anabolic and inflammatory aspects using bovine articular chondrocytes [149]. CS inhibited IL-1β-induced expression of the pro-inflammatory genes iNOS and cyclooxygenase-2 and restored TGF-β receptors I and II mRNA levels, whereas no CS-induced decrease of NO was observed. Thus, CS may exert both chondroprotective and anti-inflammatory limited effects on articular cartilage.

Although clinical trials to examine the effect of CS on the relief of pain from OA have been carried out and reported information on this aspect with a general trend in favor of CS [141, 142], the effect of CS is not settled and some reports concluded that CS is not significantly better than a placebo [153]. Since, until now, no synthetic preparation of CS was available, all the CS ever used was extracted and purified from natural products. In some cases, little attention was paid to the structural heterogeneity of CS/DS chains. The structure of CS/DS varies depending on the source of the animal species, tissue, age, physiological conditions, and so on. Representative disaccharide compositions of CS/DS variants are shown in Table **4**. The different effects of CS on clinical trials might depend on the origin of CS preparations [154]. CS possibly has a huge variety of structures, and the physicochemical and biochemical features of CS preparations are not exactly the same. Therefore, it was not a big surprise to find major differences in terms of quality or quantity in different brands sold as over-the-counter substances [160]. Since the effect of CS on the improvement of joint function in OA patients might rely on its fine structure, the activity of CS preparations is also dominated by the presence of such fine structures. CS preparations, which have been used clinically in every day practice and in the various clinical trials performed so far, might differ in efficacy depending on their origins. For instance, the ratio of A and C disaccharide units as well as the proportion of highly sulfated disaccharide units, D and E, should be considered in the CS preparations used for treating OA. If the disaccharide composition of CS preparations varies in different batches, stable effects on the treatment of OA would not be reproduced.

There are still unanswered questions regarding the structure-function relationship of CS to control the symptoms of pain and to increase the overall mobility of patients suffering from OA. Thus, it is important to identify the fine structure of the active CS domain for the treatment of OA. Since the excellent safety and tolerability of oral CS have been confirmed [133], if this structure is clearly elucidated, CS will become more useful in the treatment of OA.

## III. ANTI-TUMOR DRUGS

Recent studies have led to great progress in the comprehension of the involvement of GAGs and PGs in the modulation of tumor cell behavior, tumor progression, and metastasis. Many studies concerning the involvement of GAGs in different aspects of tumor development have been conducted with HS. On the other hand, CS/DS-PGs in the ECM have long been considered only to be an architectural support for tumor cells. However, cells undergoing movement have a much higher quantity of CS/DS than HS [161]. A clear correlation between the accumulation of CS/DS-PGs and cancer progression has been demonstrated, although the biological role of CS/DS-PGs in the progression has not been determined. Tumor stroma and tumor fibrotic tissues contain abnormally high concentrations of CS-PGs,

**Table 4. Disaccharide Composition of CS/DS Variants**

| Sources | Refs. | Proportions of Disaccharide Units[a] (%) | | | | | |
|---|---|---|---|---|---|---|---|
| **CS** | | **O** | **A** | **C** | **D** | **B** | **E** |
| Whale Cartilage | [123] | 2 | 77 | 20 | 1 | 0 | 0 |
| Shark Cartilage | [123] | 1 | 20 | 70 | 8 | 0 | 1 |
| Shark Fin Cartilage | [123] | 2 | 35 | 40 | 22 | 0 | 1 |
| Squid Cartilage | [123] | 4 | 22 | 10 | 0 | 0 | 64 |
| Skate Cartilage | [154] | 3 | 43 | 39 | 13 | 1 | 1 |
| Bovine Lung | [155] | 3 | 42 | 54 | 2 | 0 | 0 |
| Bovine Trachea | [156] | 9 | 51 | 40 | 0 | 0 | 0 |
| Porcine Trachea | [156] | 3 | 82 | 15 | 0 | 0 | 0 |
| Chicken Trachea | [157] | 13 | 69 | 18 | 0 | 0 | 0 |
| **CS/DS[b]** | | **O/iO** | **A/iA (A iA)[c]** | **C/iC** | **D/iD** | **B/iB** | **E/iE** |
| Porcine Skin | [158] | 2 | 71 (2 69) | 1 | 2 | 24 | 0 |
| Porcine Cartilage[d] | [158] | 4 | 78 (35 43) | 11 | 4 | 1 | 1 |
| Embryonic Pig Brain | [17] | 20 | 45 (36 9) | 32 | 2 | 0 | 1 |
| Adult Pig Brain | [17] | 4 | 83 (82 1) | 9 | 2 | 0 | 2 |
| Pig Mucosa | [156] | 2 | 76 | 9 | 0 | 5 | 8 |
| Bovine Lung | [155] | 15 | 63 | 9 | 1 | 12 | 0 |
| Bovine Heart | [155] | 4 | 61 | 20 | 2 | 13 | 0 |
| Bovine Liver | [159] | 0 | 77 | 6 | 0 | 9 | 8 |
| Shark Liver | [20] | 6 | 33 (17 16) | 32 | 4 | 7 | 18 |
| Shark Skin | [18] | 10 | 41 | 33 | 3 | 0 | 13 |
| Hagfish Notochord[d] | [15] | 5 | 38 | 13 | 0 | 0 | 40 |

[a]For abbreviations used for the disaccharide units, see Fig. (**2**).
[b]The sum of the proportions of CS and DS disaccharide units in CS/DS hybrid chains is summarized.
[c]The proportions of A and iA units are shown in the parentheses.
[d]Hagfish notochord CS and porcine cartilage CS contain 4 and 1%, respectively, trisulfated disaccharide unit [HexA(2-*O*-sulfate)-GalNAc(4,6-*O*-disulfate)].

especially versican and decorin [162, 163]. This is accompanied by alterations in the type of GAG side chains by specific structural modifications. The DS on these core proteins in colon adenocarcinoma was replaced by CS chains, the molecular size of GAG chains decreased, and the sulfation pattern altered [164]. Disaccharides containing 4-*O*-sulfated GalNAc (A unit), which predominate in normal tissues, become minor constituents in malignant tumors, but amounts of nonsulfated disaccharide units (O units) and disaccharides containing 6-*O*-sulfated GalNAc (C units) are elevated. In invading melanoma, the accumulation of CS is associated with the appearance of a larger quantity of E unit [165]. These results suggest that CS/DS chains play some role in tumor progression.

CS/DS-PGs are implicated in tumor growth and progression. CS/DS-PGs have been shown to interact with many different soluble and membrane-associated molecules. The binding of a variety of growth factors and cytokines to CS/DS has been described (Table **1**). As shown in the right column of Table **1**, most of the CS/DS-binding growth factors are involved in cancer or tumor formation. Fine structural alterations of CS/DS chains in a tumor would modify the activities of effective protein factors and influence the biology of cancer cells, contributing to cell growth and migration. Oversulfated CS-E specifically interacts with various Hep-binding growth factors including FGF-2, FGF-10, FGF-16, FGF-18, vascular endothelial growth factor (VEGF), midkine (MK), PTN, and HB-EGF [14], which dictate various biological processes during tumor growth and spreading. The kinetic constants of these interactions suggest that the intensity of binding of MK, PTN and FGF-18 toward CS-E was stronger than that for Hep [14]. CS/DS from embryonic pig brain also bind to FGF-2, FGF-10, FGF-18, MK, and PTN [17, 120]. CS-H or DS-E rich in iE units from hagfish notochord interacts with FGF-2, FGF-10, FGF-16, FGF-18, glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), VEGF, and HB-EGF [15]. DS chains enhance FGF signaling through interactions with FGF-2 and FGF-7 during wound repair [166]. DS chains also promote HGF/SF-induced cell proliferation [128]. CS chains of hybrid proteoglycans, syndecans -1 and -4, bind to PTN, MK, and FGF-2, cooperating with their HS chains [167]. These findings imply that CS/DS chains also function as coreceptors for growth factors. Notably, MK and PTN are overexpressed in various malignant tumors and are markers for carcinoma [168], suggesting that CS/DS chains play a crucial role in MK-mediated cell migration and PTN-mediated angiogenesis in tumor tissue. CS-GAGs have also been identified as major P-selectin-reactive ligands on the cell surface in human metastatic breast cancer cell lines and involved in P-selectin-mediated adhesion of the cells to murine platelets and human umbilical vein endothelial cells [169].

Although the specific interaction between CS/DS and a number of functional proteins has been demonstrated (Table **1**), information on the precisely sulfated structures required for the binding is limited (Table **2**). Maimone and Tollefsen [12] determined the structure of a DS hexasaccharide that binds to heparin cofactor II with high affinity. Kawashima *et al.* [30] reported that a tetrasaccharide containing E units interacts with L- and P-selectins and some chemokines. Bao *et*

*al.* [96] isolated a series of PTN-binding octasaccharides using the PTN-column after partial fragmentation of the CS/DS hybrid chains from embryonic pig brain, and found that multiple binding sequences containing D or iD units may be relevant for PTN-binding.

Although the greater part of the sequences for the binding of CS/DS to effective molecules have not been determined, such specific sequences are molecular targets for cancer diagnosis and therapeutics. Detection of CS/DS oligosaccharide sequences specific for malignant tumor cells will be instrumental for identification of malignancy of cancer. Recently, antibodies recognizing CS/DS oligosaccharide sequences were reported. An antibody WF6, raised against shark aggrecan CS-PG, specifically recognizes an epitope in CS chains. This epitope was investigated using an oligosaccharide microarray, and two binding CS octasaccharide sequences were identified [97]. The serum concentration of the WF6 epitope is highly increased in ovarian cancer [170]. Thus it may provide a useful biomarker for cancers and other disorders of the ovary. ten Dam *et al.* [171] selected a phage display antibody GD3G7 reacting with a CS variant rich in the E-unit (CS-E). To characterize the epitope of GD3G7, structurally defined octa- and decasaccharide fractions derived from CS-E were used in an inhibitory enzyme-linked immunosorbent assay, and it was found that GD3G7 recognizes decasaccharides with a minimum of three consecutive E units [172]. The epitope of this antibody is strongly expressed in ovarian adenocarcinomas and implicated in the formation of tumor blood vessels by VEGF signaling. CS-E binds to VEGF, and the antibody GD3G7 can compete with the binding. Thus, the specific CS sequence recognized by GD3G7 might be a potential target to suppress tumor development. Likewise, CS/DS oligosaccharides with specific properties to bind effective molecules on tumor progression may inhibit the metastasis and proliferation of the tumor cells by neutralizing the activities of growth factors and cell adhesion molecules. The detection and identification of such CS/DS saccharide sequences will make an important contribution to cancer therapy.

The expression of CS/DS is often unregulated in cancer cells, indicating that CS/DS are connected with cell kinesis. The control of CS/DS expression by the modulation of metabolism may help in the research of a novel anti-tumoral strategy. Regulation of the appropriate enzymes involved in the biosynthesis and degradation of CS/DS using recent technologies to govern gene expression may affect tumor growth. Some other novel applications have also been reported using CS/DS. Pumphrey *et al.* [173] showed that the injection of a carbodiimide-modified CS directly into breast tumors growing in nude mice reduced or abolished cancer-cell growth without causing apparent toxicity to the adjacent normal tissue. Borsig *et al.* [174] recently demonstrated that fucosylated CS isolated from sea cucumber inhibited the attachment of LS180 carcinoma cells to immobilized P- and L-selectins as well as inhibited lung colonization by adenocarcinoma MC-38 cells in an experimental metastasis mouse model, suggesting that invertebrate CS may be a potential alternative to Hep for blocking metastasis and inflammatory reactions without undesirable anticoagulant side effects. As described above, specific CS-PGs markedly accumulate in various types of cancer. Taking into account the observation that CS is overexpressed in several highly metastatic tumors, CS may well be used as a target for the selective delivery of anticancer drugs by polyethylene glycol-coated liposomes [175]. These liposomes contain a new cationic lipid 3,5-dipentadecycloxybenzamidine hydrochloride bound preferentially to CS and avidly internalized by highly metastatic tumor cells. When these liposomes are loaded with cisplatin, they effectively kill CS-expressing tumor cells.

## CONCLUSION

CS/DS possibly have a huge variety of structures, and the physicochemical and biochemical features of different CS/DS preparations are not the same. The structure of CS/DS molecules varies depending on the source of the animal species, tissue, age, physiological conditions, and so on. So far, little attention has been given to such differences in CS/DS chains, when CS/DS polysaccharides are administered as functional foods or drugs. However, it should be noted that the same clinical effects would not be produced unless they have a similar structure. Furthermore, such structural heterogeneity makes CS/DS chains versatile and able to specifically interact with and regulate many kinds of bioactive proteins. In most cases, however, the precisely sulfated distinct structures of CS/DS, which specifically bind to functional proteins, have not been elucidated. This is because of the structural complexity of CS/DS chains. Therefore, if the sequences of the CS/DS oligosaccharides, which regulate the functions of effective proteins such as growth factors and cytokines, are identified, the specific CS/DS sequences may become molecular targets for novel diagnosis and therapeutics of diseases related to protein factors. Until now, the application of CS/DS and their derivatives to therapeutic strategies has been limited. However, taking into account the huge variety and physiological importance of the proteins interacting with CS/DS, CS/DS are clearly very good research seeds for developing medicines. Structural elucidation of the functional domains of CS/DS is desired to obtain information for the development of new therapeutics.

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