論文の内容の要旨

応用生命化学専攻
平成 18 年度博士課程　　入学
氏　　名　　金　淼
指導教員名　清水　誠

論文題目

The suppressive effects of chondroitin sulfate and its oligosaccharides on IL-6 secretion in macrophage-like cells

（コンドロイチン硫酸およびそのオリゴ糖がマクロファージ様細胞のIL−６分泌に及ぼす抑制効果）

Chondroitin sulfate (CS) is a member of glycosaminoglycans (GAGs) which are an important family of highly functionalized, linear, and negatively charged bioactive polysaccharides that are ubiquitous components of animal connective tissue. CS is firstly isolated from cartilage and consists of repeating (1->4)-linked disaccharide units of β-D-glucuronic acid linked (1->3) to N-acetyl-β-D-galactosamine. The two most common isomers, CS-A and CS-C, are sulfated at C-4 and C-6 of the galactosamine residue, respectively (Fig. 1). Several reports have shown that CS has anti-inflammation characteristics. CS is therefore commonly used as an ingredient for dietary supplements taken as an alternative medicine to treat osteoarthritis. CS is also approved and regulated as a symptomatic slow-acting drug for this disease (SYSADOA) in Europe and some other countries.

The recently research about immune functions of CS has been focused on its anti-inflammatory effects. But the signaling pathways and mechanism associated with the action of CS in cells still haven’t been clearly defined. The pro-inflammatory responses induced by the microbe compounds via toll-like receptors (TLRs) in macrophages are characterized by secretion of cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6, etc. Recent studies have documented a series of IL-6 activities that are critical for resolving innate immunity and promoting adaptive immune responses. Overproduction of IL-6 leads to inflammation and diseases such as rheumatoid arthritis and Crohn's disease. In this study, we focused on the suppressive effects of CS and its oligosaccharides on the IL-6 secretion induced by TLR ligands in macrophage-like cells. And also the permeation mechanism of disaccharides derived from CS (Di-CSs) (Fig. 1) has been investigated.

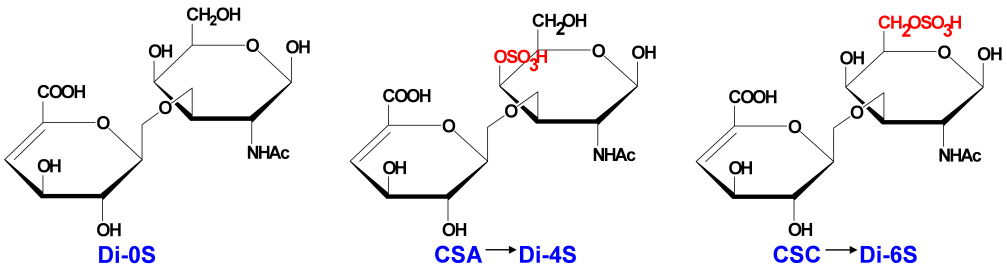

Fig. 1. Structures of CS disaccharides (Di-0S, Di-4S and Di-6S). Di-4S, the disaccharide unit of CS-A, is sulfated on C-4 of the galactosamine while Di-6S, the disaccharide unit of CS-C, is sulfated on C-6 of the galactosamine. Whereas Di-0S carries no sulfated group.

Chapter 1. The permeation study of Di-CSs across human intestinal Caco-2 cell monolayers

Although CS has caused widespread interest in food and pharmaceutical industries, the intestinal absorption of CS has been controversial due to its high molecular weight and charge density. So far, a variety of analytical methods have demonstrated that CS can be absorbed by oral route. Furthermore, lots of studies showed that the structure and molecular size of CSs strongly influence their absorption and bioavailability, but the detailed absorption mechanism of CS still has not been revealed. In this study, CS was enzymatically hydrolyzed to prepare disaccharides (Di-CSs), and their intestinal permeability was investigated by using monolayers of intestinal Caco-2 cells which have been widely used to study the transport mechanisms of drugs. The amount of permeated disaccharides was determined by strong anion-exchange high-performance liquid chromatography (SAX-HPLC). Treatment of Caco-2 cell monolayers with sodium azide (NaN_3), a metabolic inhibitor, did not affect the transepithelial transport of Di-4S and Di-6S through Caco-2 cell monolayers, suggesting that active transport is not involved in the transport of Di-CSs.

Interferon-gamma (IFN-γ) and Cytochalasin (Cyto) B are known to increase the paracellular passive diffusion across the intestinal epithelial cell monolayers by altering the cytoskeletal structure which results in opening tight junctions. The permeabilities of Di-4S and Di-6S were remarkably increased when the transepithelial electrical resistance (TER) value of Caco-2 cell monolayers was dropped markedly by Cyto B or IFN-γ treatment. These results suggested that the most plausible mechanism for Di-CSs permeation through Caco-2 cell monolayers is paracellular diffusion.

The three different types of authentic Di-CSs, Di-4S, Di-6S and Di-0S (Fig. 1), were also introduced in this study to investigate whether the sulfur group would affect the transepithelial transport of CS disaccharides. Our data indicated that the permeability of the three different disaccharides was not significantly different. This may suggest that the sulfur group is not the key factor determining the transepithelial transport of Di-CSs.

Chapter 2. The effects of CS and its oligosaccharides on IL-6 secretion induced by TLR ligands in macrophage-like cells

The immune-modulating effects of CS and its oligosaccharides were concentrated on TLR-mediated inflammation in a macrophage-like cell line J774.1. Secretion of IL-6 from J774.1 cells stimulated by Pam3CSK4, Poly (I:C), LPS and CpG, which is the ligand of TLR1/TLR2, TLR3, TLR4 and TLR9 respectively, was investigated by co-treatment with CS or its oligosaccharides.

The disaccharide of CS-A, Di-4S, suppressed the IL-6 secretion induced by all four TLR ligands. Di-6S also significantly suppressed the IL-6 secretion induced by Pam3CSK4, Ploy I:C and CpG. The

strongest suppressive effect on IL-6 secretion was observed when cells were stimulated by CpG; except CS-A, all other molecular types and sizes of CS can markedly suppress IL-6 secretion. Di-4S and Di-6S also significantly suppressed CpG-induced IL-6 secretion in another macrophage-like cell line RAW 264.

Furthermore, our experimental data suggested that CpG-induced IL-6 suppression in J774.1 caused by CS-A was molecular size-dependent; the smaller sized CS-A exerted more significant IL-6 suppression. However, similar size-dependency was not observed by CS-C treatment. Our results also pronounced the structure-dependent IL-6 suppression of CS and Di-CSs while J774.1 cells were stimulated by CpG. Between the two intact CSs utilized in our study, CS-C presented significantly stronger IL-6 suppression than CS-A. Interestingly, among the three types of Di-CSs with different sulfation (Fig. 1), Di-4S showed markedly stronger IL-6 suppressive effect than Di-6S and Di-0S. Similar structure-dependent IL-6 suppression caused by Di-CSs was also observed while J774.1 cells were induced by LPS, Pam3CSK4 and Poly:IC. Although the sulfur group of CS was thought to play an important role in its immune-modulating behavior, non-sulfated Di-0S showed close IL-6 suppression compared with Di-6S. This suggested that the sulfate residue is not always essential for the activity, but position of the sulfate residues may be important.

The results presented in this chapter indicated that characteristics like molecular size and structure are likely to be the determinants which govern the immune-modulating functions of CS and its oligosaccharides, especially on the TLR-related inflammatory responses.

Chapter 3. The mechanism study of CSs/Di-CSs on CpG-induced IL-6 suppressive effects in marcophage-like J774.1 cells

CS-C and Di-CSs also suppressed CpG-induced IL-6 mRNA expression, suggesting that the action point of CS-C and Di-CSs in suppressing CpG-induced IL-6 secretion exists at a stage earlier than the IL-6 gene transcription (Fig. 2B). CpG stimulated the activation of TLR9 to trigger the molecule myeloid differentiation primary response gene (88) (MyD88)-dependent inflammatory signaling

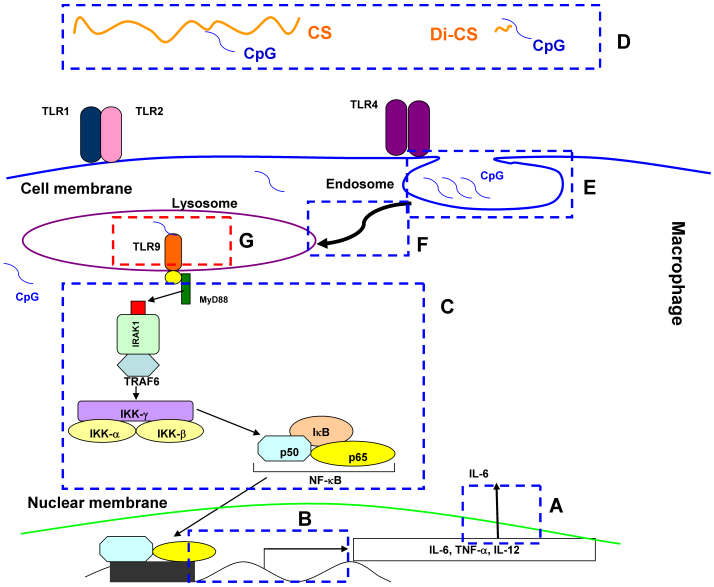

Fig. 2. Schematic diagram of possible action points of CSs/Di-CSs which suppressed the CpG-induced IL-6 secretion in macrophage J774.1 cells. Part A: IL-6 translation, Part B: IL-6 transcription, Part C: signaling pathway after TLR9 activation, Part D: direct binding of CSs/Di-CS with CpG, Part E: phagocytosis of CpG by J774.1 cells, Part F: CpG traffic to lysosome, Part G: CpG recognized by TLR9.

pathway. The effect of CSs/Di-CSs on the CpG-induced degradation of IL-1 receptor-associated kinase 1 (IRAK1), which was involved in the early stage of MyD88-dependent inflammatory signaling pathway, was therefore examined. IRAK1 degradation was clearly inhibited by Di-4S, Di-6S and CS-C, indicating this inhibition to be a possible mechanism point of the IL-6 suppression by CS (Fig. 2C). However, some steps that precede the signaling pathway triggered by TLR9 can also switch off the downstream signaling activation. Several other action points of CSs/Di-CSs preceding the signaling pathway by TLR9 activation were therefore checked in this study (Fig. 2D-G). Results of size exclusion chromatography (SEC) indicated that CSs/Di-CSs couldn't directly bind with CpG (Fig. 2D). Flow cytometry results of fluorescein isothiocyanate (FITC)-labelled CpG demonstrated that CSs/Di-CSs couldn't affect the phagocytosis of CpG by J774.1 cells (Fig. 2E) which was necessary for CpG recognition by TLR9 and triggered the IL-6 inflammatory signaling pathway. Immune fluorescence results suggested that oligosaccharides derived from CS did not affect CpG traffic to TLR9 (Fig. 2F), although CSs appeared at the same location of CpG and TLR9. Finally, results of immunoprecipitation suggested that Di-4S interfered with the CpG-TLR9 interaction (Fig. 3G), which would be one of the additional mechanisms.

CS has been reported to directly bind with mannose receptor in a structure-dependent manner. The similar CS binding domain of mannose receptor also exists in TLR9. Thus, it is probable that CSs/Di-CSs directly bind with TLR9. The competition of CpG and CSs/Di-CSs in binding with TLR9 may affect the interaction of TLR9 and CpG, and therefore weaken the stimulation of IL-6 signaling pathway by CpG. It would be also possible that CSs/Di-CSs play as antagonistic ligands of TLR9, thereby suppressing the IL-6 signaling pathway activation.

Understanding the possible permeation mechanism of Di-CSs would be beneficial to improve their bioavailability and also be helpful to exert their immune functions. With enough evidence and knowledge that Di-CSs can be actually absorbed in the oral route, further interesting immune research on CS would be worthy to be expected.

Our results have suggested that CS-C and Di-CSs would be effective suppressing agents for TLR9-mediated inflammation in macrophages. Until now, there was no report describing the natural substances which suppress the CpG-induced inflammatory reaction. The immune-modulating effects of CS-C and Di-CSs presented in this study will be helpful to understand the anti-inflammatory mechanism of CS and its oligosaccharides and other sulfated polysaccharides, especially in terms of the TLR-mediated inflammatory responses.

Publications

1. Jin, M., Yamada, K., Satsu, H., Hisada, N., Totsuka, M. and Shimizu, M., The modulating effects of chondroitin sulphate A and its oligosaccharides on a macrophage-like cell line J774.1. *J. Clin. Biochem. Nutr.*, 43(supp.1), 370-373 (2008)

2. Jin, M., Iwamoto, T., Yamada, K., Satsu, H., Totsuka, M. and Shimizu, M., Disaccharide derived from chondroitin sulfate A suppressed CpG-induced IL-6 secretion in macrophage-like J774.1 cells. *Cytokine*, accepted