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Discovery of a TNF-α Antagonist Using Chondroitin Sulfate Microarrays

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Chondroitin sulfate (CS) glycosaminoglycans play important roles in biological processes, such as neural development, viral invasion, cancer metastasis, and spinal cord injury.[1] The diverse sulfation patterns of CS polysaccharides have been postulated to function as molecular recognition motifs for growth factors, chemokines, and other proteins.[1a,2] However, no method currently exists to rapidly identify CS-binding proteins or specific sulfation motifs involved in protein recognition. Carbohydrate microarrays have been used extensively to characterize glycan−protein interactions,[3] but they have not been exploited for detailed structure−function studies of glycosaminoglycans. Moreover, application of carbohydrate microarrays has been limited largely to confirming known interactions with well-characterized lectins, proteins, and antibodies.[3a−c] Here, we report the first example of synthetic CS microarrays, and we use these microarrays to identify a previously unknown interaction between chondroitin sulfate-E (CS-E) and tumor necrosis factor-α (TNF-α).

The three major sulfation motifs found in vivo, CS-A, CS-C, and CS-E,[4] differ only subtly in their sulfation pattern and are identical in terms of stereochemistry and sugar composition (Figure 1). Carbohydrate microarrays should provide a powerful approach to evaluate the importance of sulfation in modulating protein recognition. However, the potential of microarrays to distinguish such closely related structures was unclear prior to this work, as most studies have utilized carbohydrates of very different composition, such as mannose versus galactose or tetrasaccharides versus hexasaccharides.[3]

To create the microarrays, we designed a general, highly efficient strategy to attach synthetic oligosaccharides to the array surface. CS molecules displaying different sulfation sequences were synthesized with an allyl functionality on the reducing end of the sugar (Figure 1).[5] This group is stable to the chemical manipulations used to synthesize the oligosaccharides, yet it can be readily functionalized for surface conjugation. Ozonolysis of compounds **1**−**4** followed by treatment with 1,2-(bisaminooxy)ethane furnished CS oligosaccharides with a convenient aminooxy handle for covalent attachment to aldehyde-coated glass slides. Importantly, this strategy requires minimal manipulation of the sulfated oligosaccharides, enabling their direct conjugation in two short, high-yielding steps. Moreover, the approach is compatible with standard DNA robotic printing and fluorescence scanning technology, which requires only minimal amounts of material and allows a large number of molecular interactions to be probed simultaneously.

We validated the approach using antibodies selective for specific CS sulfation motifs. A high-precision contact-printing robot was used to deliver nanoliter volumes of the compounds to the slides, yielding 1000 spots approximately 200 μm in diameter. Unreacted aldehyde groups were quenched with $NaBH_4$ prior to use. The microarrays were incubated with monoclonal antibodies raised against CS-A tetrasaccharide **1** or CS-E tetrasaccharide **3** conjugated to keyhole limpet hemocyanin, and antibody binding was visualized using a secondary Cy3-conjugated goat anti-mouse antibody. The

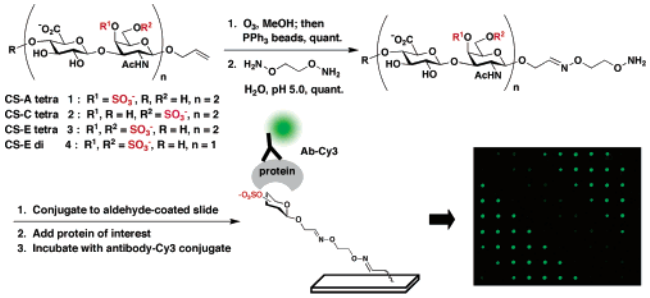

Figure 1. Conjugation of CS oligosaccharides to microarray surface and strategy for analysis of CS−protein interactions.

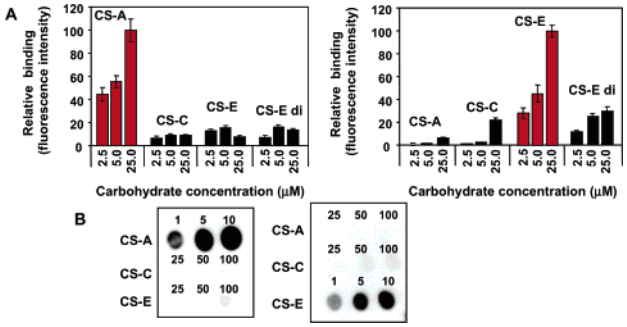

Figure 2. (A) Binding analysis of the CS-A (left) or CS-E (right) antibody to the microarrays. Each bar represents the average of 10 points. (B) Dot blots depicting binding of the CS-A (left) or CS-E (right) antibody to CS oligosaccharide−BSA conjugates (ng).

CS-A antibody bound to the CS-A tetrasaccharide in a concentration-dependent manner, and strong selectivity for the CS-A motif was observed, with little detectable binding to the CS-C or CS-E sulfation motifs (Figure 2A). Similarly, the CS-E antibody selectively recognized the CS-E tetrasaccharide and displayed only weak binding to the CS-C motif at high tetrasaccharide concentrations. To examine the carbohydrate chain length required for interaction, we compared the ability of the CS-E antibody to bind CS-E di- and tetrasaccharides. The CS-E disaccharide showed significantly reduced antibody binding, indicating a clear preference of the antibody for tetrasaccharide epitopes.

To confirm the antibody specificities obtained from the microarrays, we performed traditional dot blot analyses. Compounds **1**−**3** were covalently attached to bovine serum albumin (BSA) by oxidation to the corresponding aldehydes, followed by reductive amination to link the carbohydrates to lysine residues of the protein. The CS−BSA conjugates were spotted onto nitrocellulose membranes and incubated with the CS-A or CS-E antibody. Antibody binding was visualized by chemiluminescence using a secondary goat anti-mouse antibody conjugated to horseradish peroxidase. Consistent with the microarray data, highly selective binding of the antibodies to their respective sulfated antigens was observed (Figure 2B).

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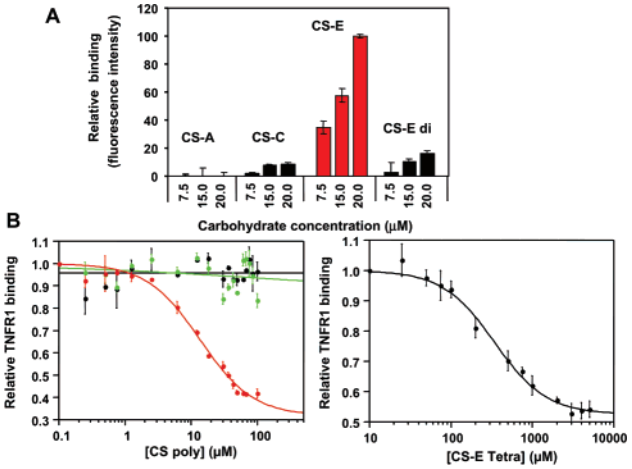

Figure 3. (A) TNF-α selectively binds to CS-E tetrasaccharide **3** on the microarray. (B) CS polysaccharides enriched in the CS-E motif (left) and CS-E tetrasaccharide **3** (right) inhibit TNF-α binding to TNFR1. Polysaccharides enriched in CS-A (black), CS-C (green), and CS-E (red) are compared on the left.

Having shown that microarrays can be exploited to identify specific sulfation motifs involved in protein recognition, we turned to the identification of novel CS-binding proteins. Previous studies have shown that CS interacts with growth factors and plays a role in inflammation and injury.[1a,c,6] Thus, we examined whether CS could bind to TNF-α, a proinflammatory cytokine involved in numerous diseases, including rheumatoid arthritis, Crohn's disease, and psoriasis.[7] The CS microarray was incubated with TNF-α, and binding was detected using an anti-TNF-α antibody followed by a secondary Cy3-labeled antibody. Notably, selective binding of TNF-α to the CS-E tetrasaccharide was observed on the microarray, with little or no binding to the CS-A or CS-C motifs (Figure 3A). As a negative control, we showed that fibroblast growth factor-1 (FGF-1), which does not interact with CS,[2] displayed no appreciable binding to the carbohydrate microarray (Supporting Information). These results represent the first binding studies using well-defined CS molecules and demonstrate the ability of distinct sulfation motifs to direct molecular recognition events.

We next examined whether CS-E could agonize or antagonize the binding of TNF-α to the cell surface receptor, TNFR1. TNFR1 was immobilized on a microtiter plate, and binding of TNF-α to the receptor was measured in the presence of varying concentrations of the CS-E tetrasaccharide or naturally occurring CS polysaccharides. Both CS-E tetrasaccharide **3** and polysaccharides enriched in the CS-E sulfation motif inhibited the interaction between TNF-α and TNFR1 (Figure 3B). In contrast, polysaccharides enriched in the CS-C or CS-A motifs could not antagonize the TNF−TNFR1 interaction. Potency measurements showed a median inhibitory concentration (relative IC_{50}) for the CS-E polysaccharide of 13.7 ± 2.5 μM, which is comparable to a recently reported small molecule inhibitor of TNF-α.[8] Although the potency of the tetrasaccharide (relative IC_{50} of 343.9 ± 37.8 μM) was reduced relative to the polysaccharide, the activity of the two compounds is comparable (25-fold difference) given that the polysaccharide is estimated to contain 37 CS-E tetrasaccharide epitopes. Notably, the IC_{50} values are within the physiological concentration range of CS, which is estimated to be at least 60 μM in the brain and may exist at 5- to 10-fold higher local concentrations at the cell surface and in the extracellular matrix.[9]

The ability of CS-E to disrupt the TNF−TNFR1 interaction suggested that CS-E might inhibit TNF-α-induced cell death. Histiocytic lymphoma U937 cells were treated with TNF-α and varying concentrations of tetrasaccharide **3** or CS polysaccharides

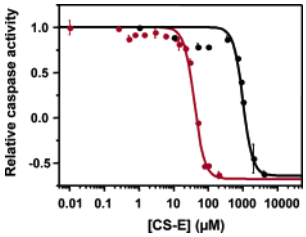

Figure 4. CS polysaccharides enriched in the CS-E motif (red) and CS-E tetrasaccharide **3** (black) inhibit TNF-α-induced apoptosis. See Supporting Information for details.

enriched in the CS-E sulfation motif. The extent of apoptosis was determined by monitoring the production of caspases 3 and 7 using a fluorescent rhodamine−DEVD peptide substrate. Both compounds prevented the cells from undergoing apoptosis, effectively blocking cell death (Figure 4). Interestingly, treatment of the cells with high concentrations of either compound reduced the extent of cell death compared to cells not treated with TNF-α, suggesting that the compounds may exert a protective function.

In conclusion, we report the first example of carbohydrate microarrays to rapidly identify glycosaminoglycan−protein interactions and probe the specificity of proteins for distinct sulfation sequences. Using the microarrays, we discovered a novel interaction between CS and TNF-α and demonstrated that CS-E tetra- and polysaccharides can antagonize the activity of this therapeutically important cytokine. The specificity of this molecular interaction is intriguing given the lack of small molecule inhibitors of TNF-α[7c,8] and the prevalence of CS glycosaminoglycans at sites of inflammation.[6] We anticipate that CS microarrays will accelerate our understanding of glycosaminoglycan−protein interactions and the role of sulfation in modulating physiological and disease states.

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Supporting Information Available: Experimental procedures for microarray production and analysis, antibody generation, dot blot, TNF-α assays, and the complete ref 8. This material is available free of charge via the Internet at http://pubs.acs.org.

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