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Heparin disaccharides inhibit tumor necrosis factor-α production by macrophages and arrest immune inflammation in rodents

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Abstract

Inflammation is the clinical expression of chemical mediators such as the pro-inflammatory cytokine tumor necrosis factor (TNF-)-α produced by macrophages and other cells activated in the immune response. Hence, agents that can inhibit TNF-α may be useful in treating arthritis and other diseases resulting from uncontrolled inflammation. We now report that the cleavage of heparin by the enzyme heparinase I generates sulfated disaccharide (DS) molecules that can inhibit the production of TNF-α. Administration of nanogram amounts of the sulfated DS molecules to experimental animals inhibited delayed-type hypersensitivity to a skin sensitizer and arrested the joint swelling of immunologically induced adjuvant arthritis. Notably, the sulfated DS molecules showed a bell-shaped dose–response curve *in vitro* and *in vivo*: decreased effects were seen using amounts of the DS molecules higher than optimal. Thus, molecular regulators of inflammation can be released from the natural molecule heparin by the action of an enzyme.

Introduction

Heparin has been noted to inhibit inflammation independent of its known anti-coagulant activity (1–4). We found, for example, that the administration of very low doses of heparin or of chemically modified heparins lacking anti-coagulant activity could inhibit delayed-type hypersensitivity (DTH) reactions in mice or adjuvant arthritis in rats (5,6). These anti-inflammatory effects showed a bell-shaped dose–response curve: inhibition was evident at relatively low doses of heparin but not at high doses. We therefore proposed that some heparin preparations might include molecules responsible for inducing leukocytes to down-regulate their inflammatory reactivities (5,6). Until now there have been no reports describing such molecules and how they might act. However, a lead into the anti-inflammatory components of heparin was provided by our studies of a related molecule, heparan sulfate.

We found that a tri-sulfated disaccharide (DS) could be cleaved from the heparan sulfate proteoglycan of the extracellular matrix (ECM) by the action of a heparinase enzyme purified from human placenta (7). This ECM-DS inhibited the DTH reactions of mice to a skin-sensitizing antigen (8). The anti-inflammatory effects of the ECM-DS were found to be associated with inhibition of production by T cells of active tumor necrosis factor (TNF)-α, a major pro-inflammatory cytokine (8). If, we reasoned, mammalian heparanase can generate from heparan sulfate an anti-inflammatory DS, then perhaps DS molecules produced by a heparin-degrading enzyme (9) might also have anti-inflammatory activities. We used the bacterial heparinase I to test this possibility; this paper reports the results.

Methods

Heparin DS molecules

Heparin sodium salt from porcine intestinal mucosa (Sigma, St Louis, MO) was solubilized at a concentration of 50 mg/ml

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in 50 mM sodium acetate containing 100 mM NaCl and 2 mM $CaCl_2$ at pH 6.5. Heparinase I (EC 4.2.2.7; Grampian Enzymes, Glasgow, UK) was added at a final concentration of 0.6 U/ml. The reaction mixture was incubated at 22°C for 18 h. Heparin degradation products were separated as described (10), using a 4.6×250 mm SAX-HPLC column (Spheriosorb; 5 μm particle size). A HPLC gradient separation was programmed using mixtures of solutions A (0.2 M NaCl) and B (1.5 M NaCl; both at pH 3.5) of 30–100% of solution B. The flow rate was 1.5 ml/min and eluted peaks were monitored at 232 nm. *O*-(α-L-ido-4-enepyranosyluronic acid 2-sulfate)-(1→4)-2-sulfamino-2-deoxy-D-glucose (α-ΔUA-2S-1→4-GIcNS; D-DS) was eluted at 4.28 min. The D-DS molecule was well resolved from the di-sulfated disaccharide *O*-(α-L-ido-4-enepyranosyluronic acid)-(1→4)-2-sulfamino-2-deoxy-D-glucose 6-sulfate (α-ΔUA-1→4-GIcNS6S) that eluted just before D-DS. *O*-(α-L-ido-4-enepyranosyluronic acid 2-sulfate)-(1→4)-2-sulfamino-2-deoxy-D-glucose 6-sulfate) (α-ΔUA-2S-1→4-GIcNS,6S; T-DS) was eluted at 6.43 min. The peaks were collected and dialyzed against de-ionized water using 100 MWCO dialysis bags (Spectrum). The mol. wt values of the sulfated disaccharides were confirmed by FAB-MS as described (11). The purity of the D-DS and T-DS molecules was >99.7% as determined by HPLC. The non-sulfated molecule *O*-(α-L-ido-4-enepyranosyluronic acid)-(1→4)-2-deoxy-*N*-acetyl-D-glucoseamin (α-ΔUA-(1→4)-GlcNAc; O-DS) and sulfated DS standards for HPLC identification of D-DS and T-DS were purchased from Sigma.

Animals

Inbred mice of the BALB/c and C57BL/6 strains were obtained from Jackson Laboratories (Bar Harbor, ME), and Lewis strain rats were supplied by Harlan Olac (Bicester, UK). Female animals were used at the age of 2.5 months.

DTH and adjuvant arthritis reactions

The DTH assay was done using groups of BALB/c mice, five mice per group, sensitized to the skin sensitizer oxazolone as described (8). The mice were sensitized on the shaved abdominal skin with 100 μl of 2% oxazolone in acetone/olive oil (4:1; v:v) applied topically. DTH reactivity was elicited 5 days later by challenge with 10 μl of oxazolone in acetone/olive oil applied topically to each side of the ear. A constant area of the ear was measured immediately before challenge and 24 h later using a Mitutoyo micrometer to determine the mean increment of ear swelling in units of 10^{-2} mm (±SE). Percent inhibition was calculated as follows: 1 – [(test mice – negative control mice)/(positive control mice – negative control mice)]×100.

The positive control consisted of a DTH reaction elicited in mice treated with saline. The negative control was the background swelling reaction produced by the oxazolone in naive, un-immunized mice. Administration of test molecules s.c. was done the day before primary sensitization to oxazolone.

To induce adjuvant arthritis, Lewis rats, five rats per group, were inoculated intradermally at the base of the tail with 0.1 ml of complete Freund's adjuvant containing 10 mg/ml *Mycobacterium tuberculosis* (H37RA; Difco, Detroit, MI) as described (5). To assess the severity of arthritis, each paw was scored clinically on a scale of 0–4, based on erythema, swelling and deformity of the joint. The total arthritis score (0–16) was obtained by adding the score of each of four joints (5). To avoid any bias in scoring, arthritis and DTH reactivities were graded by an observer ignorant of the experimental protocol.

TNF-α assay

Peritoneal exudate cells were harvested from C57BL/6 mice that had been injected 4 days previously with 1.5 ml thioglycolate (Biological Services, The Weizmann Institute, Rehovot, Israel). The cells were plated in flat-bottom 96 well-plates (Nunc, Roskilde, Denmark) in DMEM containing 10% FCS (Beit Haemek, Israel) at 10^5 cells in 100 μl/well. After 2 h of incubation in a humidified incubator (7.5% CO_2, 37°C), the non-adherent cells were washed away and 150 μl saline was added to each well containing the adherent cells, which were >90% macrophages as determined by FACScan analysis using anti-MAC-1 mAb. The test molecules were added to the culture medium at different concentrations for 20 h before stimulating the cells with lipopolysaccharide (LPS; Sigma, 1 μg/ml in culture medium containing 10% FCS) for 24 h. Active TNF-α was detected by bioassay of the culture media using TNF-α standards, as previously described (8).

Statistical analysis

Statistical significance was analyzed by Student's *t*-test and by Wilcoxon's sum of ranks test as appropriate.

Results

Production of heparin-DS molecules

Figure 1 shows the HPLC profile of molecules produced by the action of bacterial (*Flavobacterium heparinum*) heparinase I (12) on porcine heparin. We isolated and purified the molecules in the peaks marked D-DS and T-DS, and identified them as the di-sulfated α-ΔUA-2S-1→4-GIcNS (D-DS) and the tri-sulfated α-ΔUA-2S-1→4-GIcNS,6S (T-DS) molecules. The effects of a non-sulfated (0-DS) molecule, α-ΔUA-(1→4)-GlcNAc, were also studied.

D-DS and T-DS inhibit TNF-α activity

To assay the effects of the DS molecules on macrophage TNF-α, we incubated mouse peritoneal macrophages with saline or with different concentrations of T-DS, D-DS or 0-DS and stimulated the macrophages with LPS. Figure 2 shows the percent inhibition of TNF-α production relative to that produced in the presence of saline. It can be seen that both the D-DS and T-DS molecules produced a bell-shaped inhibition curve; the degree of inhibition rose with increasing concentrations of the inhibitors, inhibition tended to level off between concentrations of 1 and 10 ng/ml, and there was no inhibition at 100 ng/ml. The 0-DS molecule did not inhibit TNF-α activity.

D-DS and T-DS inhibit DTH reactivity and adjuvant arthritis

The effects of administering DS molecules on cell-mediated inflammation were studied in mice and rats. Figure 3 shows the percent inhibition of a DTH reaction produced by treating

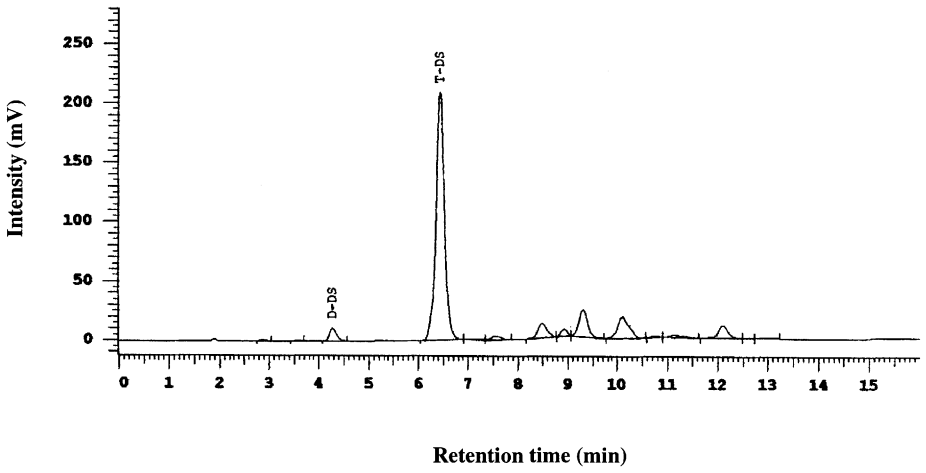

Fig. 1. SAX-HPLC profile of heparin degradation by heparinase I. Porcine heparin was degraded by incubation with heparinase I, and the products were separated using a SAX-HPLC column and monitored at 232 nm. The peaks labeled D-DS and T-DS were obtained at 4.28 and 6.43 min respectively.

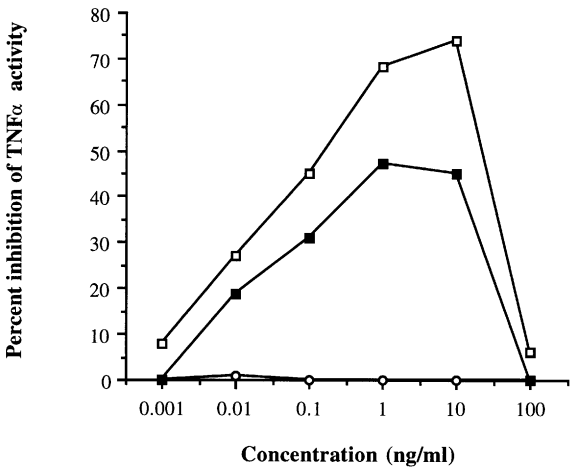

Fig. 2. Inhibition by DS molecules of TNF-α production by stimulated macrophages. Peritoneal macrophages of C57BL/6 mice were maintained in culture and incubated for 20 h with D-DS (open squares), T-DS (closed boxes) or 0-DS (open circles) at the indicated concentrations. The production of TNF-α, tested by a bioassay, was stimulated by LPS. The percent inhibition of TNF-α production was determined in comparison to cultures of macrophages incubated with saline.

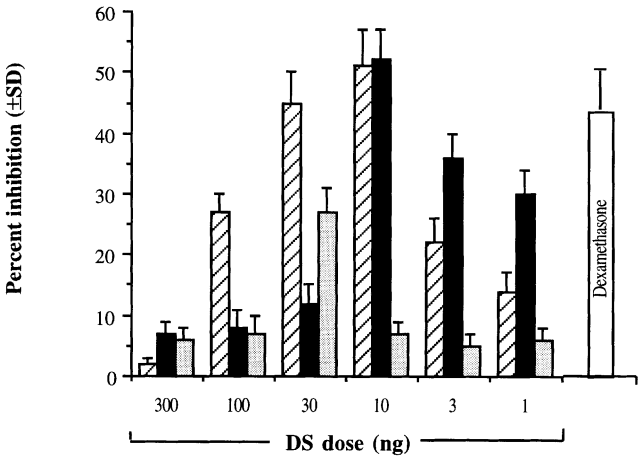

Fig. 3. Inhibition by DS molecules of a DTH reaction. Groups of BALB/c mice were sensitized to oxazolone by skin painting and the degree of DTH reactivity was assessed 6 days later by applying oxazolone to the ears and measuring the increase in ear thickness 24 h later. The mice were treated by a s.c. injection of the indicated amounts of D-DS (hatched columns), T-DS (dark columns) or 0-DS (gray columns) given 1 day before primary sensitization. Dexamethasone, 40 μg, given 1 day before challenge, was used as a positive control for an anti-inflammatory effect. The percent inhibition was computed in comparison to control sensitized mice that had been treated with saline. Percent inhibitions of 20% or more were significantly different ($P < 0.01$) from the saline control.

mice with DS molecules or with the steroid drug, dexamethasone. It can be seen that dexamethasone treatment reduced the DTH reaction by ~50%. The inhibitory effects of the DS molecules were bell-shaped: doses of D-DS or T-DS greater than the optimal doses were less effective. Treatment with 0-DS was not as effective as was treatment with the sulfated DS molecules; moderate inhibitory activity was seen only at the 30 ng dose.

To test whether the DS molecules might be effective in arresting adjuvant arthritis in Lewis rats (5), we induced the disease with *M. tuberculosis* antigen and then treated the rats 12 days later, at the outbreak of clinical swelling of the limbs. The inhibitory effects of D-DS on adjuvant arthritis manifested a bell-shaped dose–response curve; a dose of 120 ng given weekly was optimal. Both lower (30 ng) and higher (240 ng) doses were less inhibitory (Fig. 4A). The 0-DS molecule was less effective; there was no significant inhibition at doses from 30 to 240 ng (Fig. 4B).

The D-DS and T-DS molecules were effective in inhibiting adjuvant arthritis even when administered by the oral route, although higher doses of the DS molecules were required. This is illustrated in Fig. 4(C), which shows that a dose of

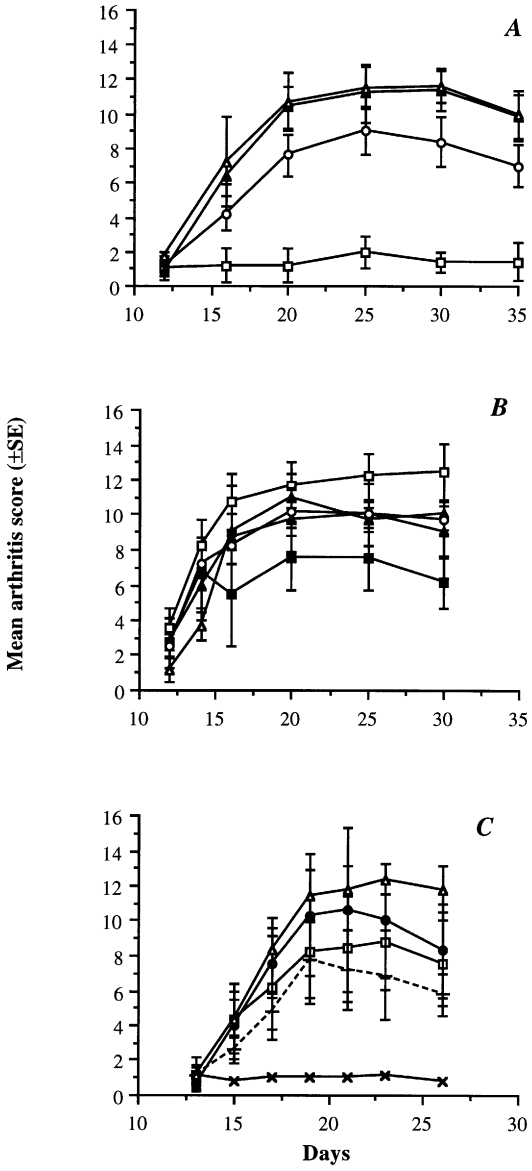

Fig. 4. Inhibition of adjuvant arthritis by DS molecules. Adjuvant arthritis was induced in groups of female Lewis rats by immunization with *M. tuberculosis* in oil. At the onset of clinical arthritis 12 days later, the rats were treated with D-DS (A), 0-DS (B) or T-DS (C) administered s.c. (A and B) or orally (C) and repeated at weekly intervals for 4 weeks. Saline control groups in each experiment (A–C) are shown as open triangles. In (A), the doses of s.c. D-DS were 30 (closed triangles), 120 (open squares) or 240 (open circles) ng. The dose of 120 ng produced significant differences from the saline control ($P < 0.01$) at days 20, 25, 30 and 35. In (B), the doses of s.c. 0-DS were 30 (closed triangles), 60 (closed squares), 120 (open squares) or 240 (open circles) ng, none of these doses affected the arthritis significantly. In (C), the doses of oral T-DS were 120 (open squares), 500 (closed circles), 1200 (crosses) or 5000 (dashed line) ng. The dose of 1200 ng produced significant differences for the saline control ($P < 0.01$) at days 17, 19, 21, 23 and 26.

T-DS of 1200 ng was effective in suppressing adjuvant arthritis. Here, too, both higher and lower doses were less effective.

The effectiveness of DS treatment was influenced by the schedule of administration. Figure 5 shows the results of an

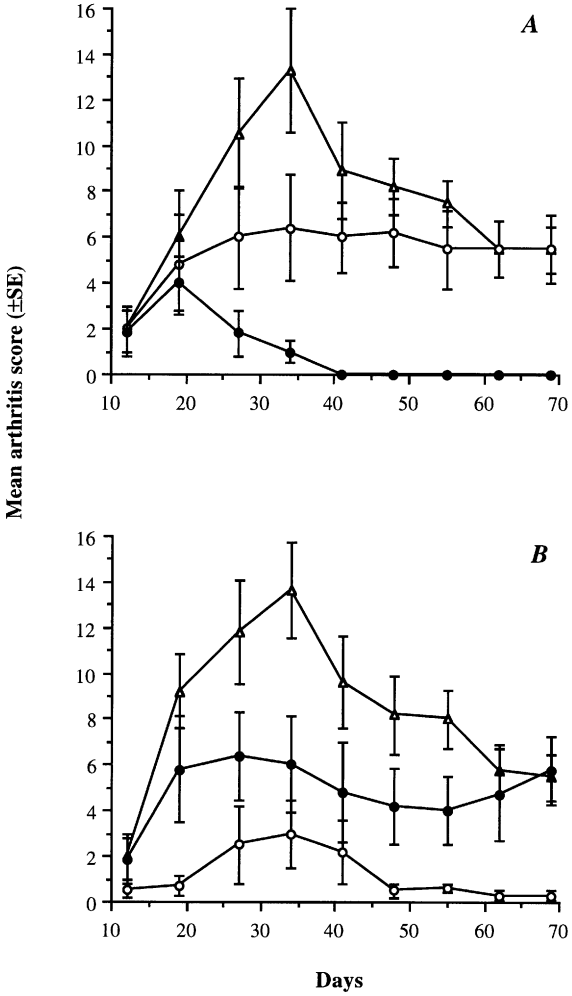

Fig. 5. Inhibition of adjuvant arthritis by D-DS or dexamethasone. Adjuvant arthritis was induced in Lewis rats as described in the legend to Fig. 4. Beginning on day 12, the rats were treated either with s.c. injections of saline (open triangles), 120 ng D-DS (closed circles) or 200 μg of dexamethasone (open circles). The treatments were repeated weekly for 4 weeks (A) or treatments were administered daily for 1 week (B). Significant differences from the saline control in (A) were $P < 0.05$ for dexamethasone at day 34, and $P < 0.01$ for D-DS at days 27, 34, 41, 49, 55, 62 and 69. In (B), the differences from the saline control were $P < 0.05$ for D-DS at day 34, and were $P < 0.01$ for dexamethasone at days 19, 27, 34, 42, 49, 55, 63 and 69.

experiment in which groups of Lewis rats received s.c. injections of D-DS at a dose of 120 ng given either weekly for 4 weeks (Fig. 5A) or daily for 1 week (Fig. 5B), beginning at the onset of clinical arthritis, 12 days after induction of adjuvant arthritis. Other groups of rats were treated with dexamethasone at a dose of 200 μg or with saline. Figure 5(A) shows that the rats treated with dexamethasone went on to develop a significant degree of arthritis that persisted despite the third and fourth weekly doses of the drug. In contrast, the arthritis in the rats treated weekly with the D-DS molecule disappeared after the fourth dose and did not reappear despite the termination of treatment. Weekly therapy with DS at 120 ng was more effective than was weekly therapy with dexamethasone at 200 μg. In contrast, dexamethasone

was more effective than was D-DS when the treatments were given daily for the first week of arthritis (Fig. 5B). Thus, both the dose of DS (Fig. 4) and the schedule of treatment (Fig. 5) appear to be important.

Discussion

The presence of heparan sulfate and related glycosaminoglycans in mast cell granules and the association of glycosaminoglycans with ECM moieties suggest the possibility that glycosaminoglycans, including heparin, might function in inflammation, wound healing and tissue homeostasis (4,13–17). However, the use of native heparin as an anti-inflammatory compound has been limited by its anti-coagulant effects (18).

Heparin is a highly sulfated copolymer with alternating residues of 1→4-linked 2-deoxy-aminoglucopyranose and hexauronic acid (15,19). Although heparin is widely used pharmacologically as an anti-coagulant and anti-thrombotic agent, its exact chemical structure has not been fully elucidated, probably because commercially available heparin preparations differ according to their tissues of origin and modes of extraction and purification (14). Nevertheless, all heparins are susceptible to degradation by *F. heparinum* heparinase I, which acts on the 1→4 glucosaminido-iduronic acid linkage and requires the presence of 2-*O*-sulfated α-L-idopyranosyluronic acid at the cleavage site (12,20).

The results presented here show that sulfated DS molecules enzymatically cleaved from heparin can down-regulate the production of TNF-α by macrophages *in vitro*, and arrest immune cell-mediated DTH and arthritis reactions *in vivo*. DS molecules added to preformed TNF-α did not inhibit the TNF-α bioassay (not shown), indicating that the DS molecules do not neutralize TNF-α but affect the production of the cytokine (8). In view of the central role of TNF-α in inflammation, it is reasonable to attribute the inhibition of immune inflammation to the inhibition of TNF-α. However, sulfated DS molecules have been reported to interact with a receptor on natural killer cells (21), and it is conceivable that D-DS and T-DS molecules might influence the physiology of inflammation by more than their effects on TNF-α production. How active DS molecules down-regulate TNF-α remains to be investigated at the molecular level. The inhibition of adjuvant arthritis by nanogram doses of the rapidly excreted DS molecules administered at weekly intervals suggests that DS may work by binding to cell surface receptors. The bell-shaped dose–response curve of activity could be explained by DS receptor de-sensitization or by the interactions of the heparin-DS molecules with functionally different receptors. We are exploring these possibilities through characterization of the proposed DS receptors. The decreased activity of the 0-DS molecule suggests that the sulfate groups of D-DS and T-DS may be functionally important. The sulfate groups of the ECM-DS molecule were also found to be important in their anti-inflammatory effects (8). It appears that the anti-inflammatory effects we noted earlier using microgram amounts of whole heparin (5,6) might be due to the presence of nanogram amounts of sulfated DS molecules in the crude mixture of molecules comprising heparin (in preparation). The use of purified heparin-DS molecules to inhibit inflammation might be advantageous because, in contrast to native heparin, DS molecules are devoid of anti-coagulant activity (2,3,9).

An important issue is the relationship between the active molecules isolated from heparin and from ECM heparan sulfate. In contrast to the D-DS and T-DS molecules derived from heparin, the tri-sulfated ECM-DS has its glucosamine moiety at the non-reducing end and its glucuronide acid moiety at the reducing end of the molecule (8–10). In addition, ECM-DS is generated by a hydrolytic enzyme, whereas D-DS and T-DS are generated by an eliminating enzyme; hence the later molecules lack the hydroxyl group on C4 at their non-reducing end. It is therefore possible that the DS and the ECM-DS might be functionally distinct. However, it is also possible that the heparin-DS and the ECM-DS molecules might be sufficiently similar to bind to the same receptors. Be that as it may, the two classes of DS molecules originate from the cleavage of distinct polymers, heparin and heparan sulfate, and are generated by different enzymes. The discovery of a mammalian heparinase enzyme with a specificity similar to that of bacterial heparinase I would suggest that DS might also be generated physiologically from heparin released in the course of inflammation. In that case, both heparin and heparan sulfate might serve as substrates for enzymes that generate breakdown products that regulate inflammation. It is also conceivable that only bacterial enzymes act to generate DS from heparin. Bacteria that generate DS from heparin might inhibit the host TNF-α response to infection, a form of regulatory signal mimicry. Thus, even if both the heparin-DS and the ECM-DS molecules turn out to be ligands for the same receptors, they would probably appear under different circumstances and may bind with different affinities. Identification of the receptor(s) for DS molecule would make it possible to sort out the relationships between these DS and help clarify the pathway leading to inhibition of TNF-α.

The results reported here suggest that DS molecules might be useful for therapeutic down-regulation of undesirable cell-mediated inflammation that characterizes certain autoimmune diseases, allergic reactions and allograft rejection. The promising anti-inflammatory effects of heparin-DS molecules may have gone unnoticed until now because of the bell-shaped dose–response curve of activity; too much material would go unnoticed.

Acknowledgements

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Abbreviations

D-DS	di-sulfated disaccharide
DS	disaccharide
DTH	delayed-type hypersensitivity
ECM	extracellular matrix
LPS	lipopolysaccharide
T-DS	tri-sulfated disaccharide
TNF	tumor necrosis factor

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