RAPID COMMUNICATION

Heparin Oligosaccharides Bind L- and P-Selectin and Inhibit Acute Inflammation

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Initial attachment of leukocytes to the vessel wall at sites of inflammation is supported by a family of carbohydrate-binding adhesion molecules called the selectins. Selectin ligands include sialyl-Lewis x (sLe^x, Neu5Acα2-3Galβ1-4[Fucα1-3]GlcNAc—) and related structures. We report here that defined heparin oligosaccharides interact with the selectins. Heparin chains containing four or more monosaccharide residues inhibited the function of L- and P-selectin, but not E-selectin, in vitro. In a competition enzyme-linked immunosorbent assay measuring inhibition of solution-phase selectin-Ig fusion proteins (selectin-Ig) binding to immobilized bovine serum albumin-sLe^x neoglycoprotein, a heparin-derived tetrasaccharide mixture inhibited 50% of L- and P-selectin-Ig binding (IC_{50}) at 200 ± 40 μmol/L and 850 ± 110 μmol/L, respectively. A single hexasulfated tetrasaccharide (ΔUA2Sα1-4IdoA2Sα1-4GlcNS6S) was particularly active against L- and P-selectin-Ig (IC_{50} = 46 ± 5 μmol/L and 341 ± 24 μmol/L). By comparison, the tetrasaccharide sLe^x was not inhibitory at concentrations up to 1 mmol/L. In cell adhesion assays, heparin tetrasaccharides reduced binding of neutrophils to COS cells expressing P-selectin but not to COS cells expressing E-selectin. They also blocked colon cancer cell adhesion to L- and P-selectin but not E-selectin. In a model of acute inflammation, intravenously administered heparin tetrasaccharides diminished influx of neutrophils into the peritoneal cavities of thioglycollate-treated mice. We conclude that heparin oligosaccharides, including non-anticoagulant tetrasaccharides, are effective L- and P-selectin inhibitors in vitro and have anti-inflammatory activity in vivo.

LEUKOCYTE (L-), platelet (P-), and endothelial (E-) selectin comprise a family of transmembrane glycoproteins whose binding to carbohydrates is fundamental to their function in cell adhesion. Each of the selectins can bind to sLe^x, a sialylated and fucosylated tetrasaccharide found as a terminal structure of sugar chains on glycoproteins and glycolipids expressed by a variety of cell types, including neutrophils, monocytes, and colon cancer cells.[1-5] Selectin binding to oligosaccharides was predicted by their N-terminal homology to the carbohydrate recognition domains of Ca^{2+}-dependent (C-type) animal lectins, such as mannose binding protein and the asialoglycoprotein receptor.[1,2]

Heparin is an unbranched, acidic glycosaminoglycan rich in N- and O-sulfate groups that is synthesized by mast cells as a component of high molecular weight proteoglycans. Heparin acts as an anticoagulant by accelerating the inhibition of thrombin and other coagulation enzymes by the circulating protease inhibitor, antithrombin (also called antithrombin III or AT-III).[6] Heterogeneous in size and extent of sulfate substitution, heparin molecules are composed of alternating residues of D-glucosamine (or N-acetyl D-glucosamine) and uronic acid (L-iduronic or D-glucuronic acid).[7] Glycosaminoglycans related to heparin are abundant components of the extracellular matrix and are present on cell surfaces in many tissues. For example, heparan sulfate proteoglycans are found on the surface of vascular endothelium where they contribute to the nonthrombogenic nature of the blood vessel lining.[8-10] Beyond its well-understood anticoagulant activity, heparin is known to bind and modulate the activity of a number of proteins, including cell growth and angiogenic factors,[11,12] complement components,[13,14] and viral proteins.[15,16] In addition, heparin has long been known to influence immunologic responses.[17-19] Recent studies have suggested that crude heparin can bind to L- and P-selectin,[20-22] and that endothelial cells contain a heparin-like ligand for L-selectin.[23] In the present study, we have quantitated the inhibition of selectin function by defined heparin oligosaccharides in vitro and tested the ability of active oligosaccharides to dampen inflammation in vivo.

MATERIALS AND METHODS

Carbohydrates. Crude heparin from porcine intestinal mucosa (average Mr = 12,000 to 15,000; cat. no. H3393), de-N-sulfated heparin (cat. no. D4776), tris-sulfated heparin disaccharide (ΔUA2Sα1-4GlcNS6S), and heparan sulfate (cat. no. H7641) were from Sigma Chemical Company (St Louis, MO). The tetrasaccharide sLe^x was from Oxford GlycoSystems, Inc (Rosedale, NY). Size-fractionated heparin oligosaccharides (Enzyme Research Laboratories, South Bend, IN) were prepared by chemical cleavage of crude porcine heparin (benzyl-esterification of carboxyl groups of iduronic acid followed by base-induced β-elimination) and multiple-

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Submitted August 10, 1993; accepted September 22, 1993.

M.P.B. is an investigator in the Howard Hughes Medical Institute and a Pew Scholar in the Biomedical Sciences. R.J.L. is funded by Grant No. GM3806 from the National Institutes of Health. O.C. is supported in part by the Experimental Pathology Program of the University of Florence, Italy.

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0006-4971/93/8210-0041$3.00/0

Table 1. Inhibition of L- and P-Selectin-Ig Binding to Immobilized BSA-sLe^x by Heparin and Related Glycosaminoglycans

Glycosaminoglycan	IC_{50} (μg/mL) L-selectin-Ig	P-selectin-Ig
Crude porcine heparin	0.4 ± 0.3 (3)	3.1 ± 0.5 (3)
LMW heparin (Mr 3,000)	28 ± 9 (3)	105 ± 20 (3)
De-N-sulfated heparin	406 ± 60 (2)	1,251 ± 6 (2)
Heparin sulfate	54 ± 13 (3)	238 ± 28 (2)

IC_{50} values were generated from competition ELISAs as described in Materials and Methods. The number of experiments for each inhibitor is shown in parentheses. Data are the mean ± SD (n = 3) or mean ± range/2 (n = 2) of calculated IC_{50} values.

step fractionation using ethanol precipitation and high-performance liquid chromatography (HPLC). A preparation of low molecular weight (LMW) heparin (average Mr = 3,000, average number of monosaccharide units = 10; Enzyme Research Laboratories) was generated by size fractionation of crude porcine heparin. The pure heparin hexasulfated tetrasaccharide (ΔUA2Sα1-4GlcNS6Sα1-4IdoA2Sα1-4GlcNS6S) was size-fractionated from enzymatically depolymerized porcine heparin using strong anion exchange HPLC, as previously described[24-27]; identity and purity were established using two-dimensional 500-MHz ^{1}H NMR, ^{13}C NMR, fast atom bombardment-mass spectroscopy, and gradient polyacrylamide gel electrophoresis.[25,28] Molecular weights used for calculation of molar concentrations were: 821, sLe^x; 1,200, heparin tetrasaccharides; and 1,330, hexasulfated tetrasaccharide.[27] Quantitation of heparin oligosaccharides by absorbance at 232 nm under acidic conditions (30 mmol/L HCl; ϵ = 5,000 (mol/L)$^{-1}$cm^{-1}) was in good agreement with quantitation based on dry weight (±5% to 20%). All monosaccharide components of carbohydrate chains except L-fucose and L-iduronic acid were in the D-configuration.

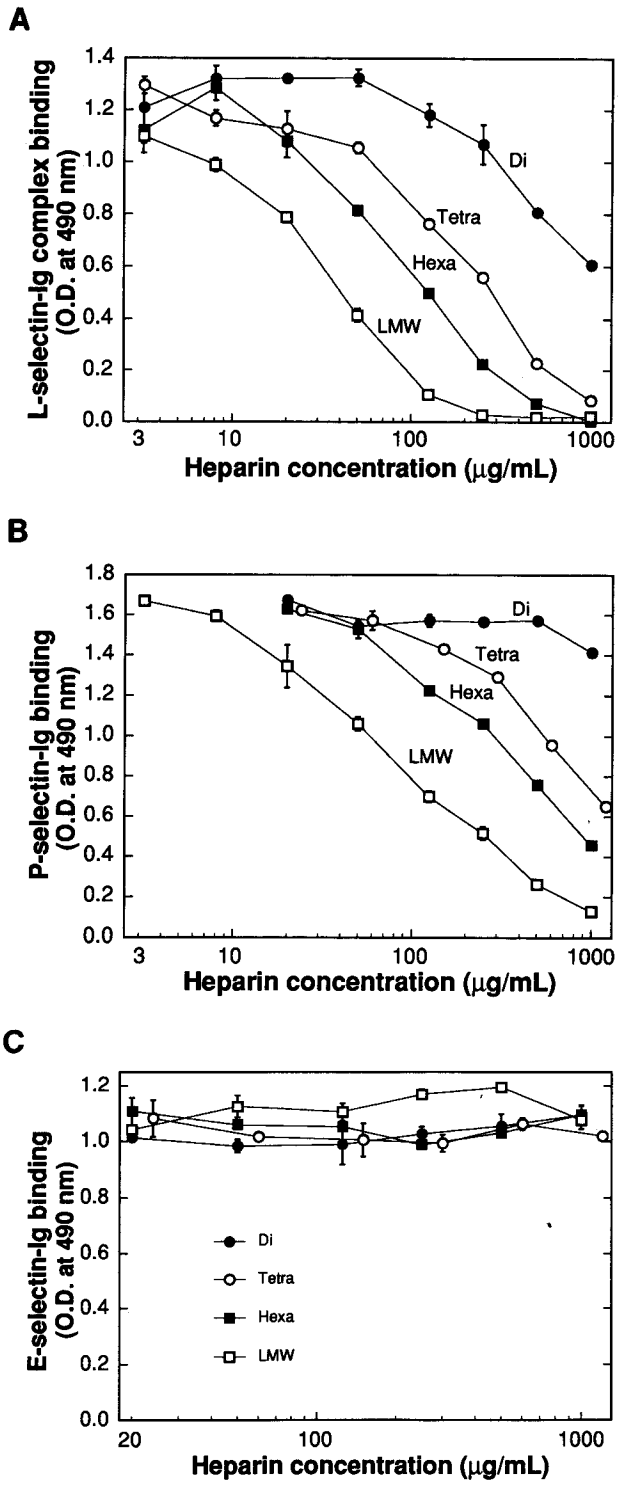
Proteins. Recombinant human selectin-Ig fusion proteins (selectin-Ig) contained the human selectin lectin domain, epidermal growth factor (EGF) module, and 1 (L-selectin-Ig), 2 (P-selectin-Ig), or 6 (E-selectin-Ig) complement regulatory modules fused to the hinge, CH2, and CH3 regions of human IgG1.[29,30] Selectin-Ig were expressed in pcDNA/COS cell[31,32] or pNUT/BHK cell[33] expression systems as disulfide-bonded dimeric molecules and purified using protein A-Sepharose or protein G-Sepharose (Pharmacia, Piscataway, NJ) affinity chromatography, as described.[32] Recombinant protein A was from Chemicon (Temecula, CA). Bovine serum albumin-sLe^x neoglycoprotein (BSA-sLe^x) was kindly provided by Chembiomed, LTD (Edmonton, Alberta, Canada). Horseradish peroxidase-conjugated goat antihuman IgG(Fc) antibody was from Jackson Immunoresearch Laboratories, Inc (West Grove, PA). BSA (Pentex Fraction V, protease-free) was from Miles Inc (Kankakee, IL). BSA-heparin neoglycoprotein was prepared using crude porcine heparin as described.[34] Heparin-Sepharose Fast Flow was from Pharmacia.

Competition enzyme-linked immunosorbent assay (ELISA). Two assays were used to measure competitive inhibition by solution-phase carbohydrates of selectin-Ig binding to immobilized BSA-sLe^x. Assays measuring inhibition of E- and P-selectin-Ig binding were performed as described,[22] except the assay buffer was 20 mmol/L HEPES pH 7.4, 150 mmol/L NaCl, 2 mmol/L $CaCl_2$, 0.25 mmol/L thimerosal, and 10 mg BSA/mL. Briefly, polystyrene microtiter plates (cat. no. 25801; Corning Glass, Newark, CA) were coated with BSA or BSA-sLe^x (0.11 μg/well in 50 mmol/L sodium carbonate/bicarbonate buffer pH 9.5) and blocked with assay buffer containing 20 mg BSA/mL. Solutions in assay buffer containing 20 nmol/L selectin-Ig and serially diluted oligosaccharides were incubated in BSA-sLe^x–coated microtiter wells for 3 hours, the wells washed, and peroxidase-conjugated goat antihuman IgG(Fc) antibody (1:3,000 to 1:15,000) was added and incubated for 30 minutes. After washing, bound selectin-Ig was quantitated by addition of chromogenic substrate for peroxidase (*o*-phenylenediamine dihydrochloride/H_2O_2) and measurement of optical density at 450 nm at 12- to 30-second intervals using a Vmax microplate reader running under Softmax software (Molecular Devices, Inc, Menlo Park, CA). An endpoint determination at 490 nm was made after stopping the color reaction in the linear range by the addition of 4 N H_2SO_4. To determine specific binding to BSA-sLe^x, signal generated by incubation of 20 nmol/L selectin-Ig in wells coated with unconjugated BSA (typically <10%) was subtracted. To enhance binding of L-selectin-Ig to BSA-sLe^x and enable competition studies, a modified version of a previously described assay was used.[35] In this assay, 20 nmol/L L-selectin-Ig was incubated together with 1:6,000 dilution of the peroxidase-conjugated antibody in assay buffer for 30 minutes before addition of inhibitors to allow formation of multimeric aggregates. After inhibitors were added, the solution was incubated an additional 30 minutes before a 2-hour incubation in BSA-sLe^x–coated microtiter wells. Subsequent washing and detection steps were the same as described above for the E- and P-selectin-Ig assays. IC_{50} values of inhibitory oligosaccharides were calculated by iterative fitting of specific binding data from individual experiments to the equation: Fraction of maximal binding = $IC_{50} \div (IC_{50} + [\text{oligosaccharide}])$, using nonlinear least-squares analysis software (Origin, Microcal Inc, Northampton, MA).

Cell adhesion assays. Cell adhesion assays on immobilized selectin-Ig fusion proteins were performed essentially as described,[22] except the test cells were LS180 colon cancer cells, which can bind to all three selectins (G. Mannori, L. Carter, O. Cecconi, K. Hanasaki, C. Corless, A. Aruffo, R. Nelson, M. Bevilacqua: manuscript in preparation). Briefly, selectin-Ig were captured (10 μL/well input: E-selectin-Ig, 5 μg/mL; P- and L-selectin-Ig, 20 μg/mL) on protein A-coated (10 μg/mL, 10 μL), BSA-blocked Nunclon Terasaki microwell plates (cat. no. 136528; Nunc, Naperville, IL). Plates were washed with Dulbecco's phosphate-buffered saline (DPBS), and 5,000 LS180 cells added to each well in the presence or absence of various heparin molecules. After 30 minutes at 4°C, nonadherent cells were removed by washing with DPBS and adherent cells fixed with 2.5% glutaraldehyde in DPBS and counted microscopically. Negative control for each experiment was adhesion in wells containing captured CD8-Ig instead of selectin-Ig (<1% of selectin-Ig adhesion); tumor cell adhesion to each selectin-Ig was inhibited using selectin-specific monoclonal antibodies (L-selectin, LAM1.3; P-selectin, G1; E-selectin, H18/7).

Adhesion of human neutrophils to COS cells transfected[31,32] with cDNA encoding full-length P- or E-selectin was assessed as follows. Neutrophils were isolated from EDTA-anticoagulated blood using sodium metrizoate-dextran gradient centrifugation (Polymorphprep; Nycomed Pharma As, Oslo, Norway) followed by hypotonic lysis of contaminating red blood cells. Transfected COS cells were cultured on gelatin-coated coverslips in 24-well culture plates (Costar, Cambridge, MA) for 36 to 60 hours after transfection. Neutrophil suspensions (10^6 cells/mL) were added (0.5 mL) and incubated at 4°C for 30 minutes. Nonadherent cells were removed by immersing the coverslips in DPBS; adherent cells were fixed to the coverslip using 2.5% glutaraldehyde in DPBS and counted microscopically. Adhesion was quantitated as number of neutrophil rosettes (three or more neutrophils on a single COS cell) per 100 transfected COS. Transfected COS cells were determined by immunofluorescent staining using selectin-specific monoclonal antibodies and a fluorescein-conjugated anti-Ig secondary antibody (Cappel, Durham, NC).

Thioglycollate-induced acute peritoneal inflammation. Mice (Balb/c males, 4 to 5 weeks old) were administered 1 mL intraperitoneal (IP) injections of 3% thioglycollate broth (lot no. 622462; Clinical Standard Laboratories, Inc, Rancho Dominguez, CA) or sterile pyrogen-free saline (Abbott Laboratories, North Chicago, IL). Ten minutes later, the animals received intravenous injections of 0.25 mL sterile pyrogen-free saline alone or containing LMW heparin or heparin tetrasaccharides (1.5 mg/mouse). Mice were sacrificed at 120 minutes and the peritoneal cavities were lavaged with 8 mL of ice-cold PBS containing 10 U/mL heparin to prevent clotting. Peritoneal cells were counted in a hemocytometer. Occasional samples contained small amounts of contaminating red blood cells that were lysed before counting. The percentage of neutrophils was assessed by counting cytospin preparations stained with Dif-Quik stain (Baxter, McGaw Park, IL; two counts per slide, 300 cells per count).



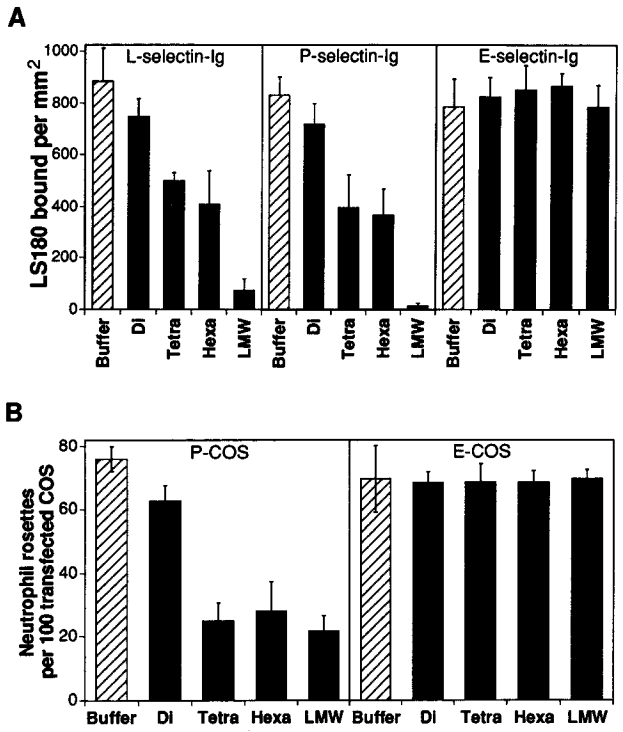


Fig 2. Effect of heparin oligosaccharides on selectin-dependent cell adhesion. (A) Adhesion of LS180 colon cancer cells to immobilized selectin-Ig fusion proteins. Data are mean and SD of adhesion in quadruplicate wells from a single experiment representative of three. (B) Human neutrophil adhesion to COS cells transfected with cDNA encoding P-selectin (P-COS) and E-selectin (E-COS). Heparins (1 mg/mL) were present (where indicated) throughout the binding incubations. Similar results were obtained in a repeat experiment. Adhesion of both LS180 cells to selectin-Ig and neutrophils to transfected COS cells could be inhibited by selectin-specific monoclonal antibodies (not shown).

Fig 1. Effect of size-defined heparin oligosaccharides on the binding of L-selectin-Ig (A), P-selectin-Ig (B), and E-selectin-Ig (C) to immobilized BSA-sLe^x (competition ELISA). Results using LMW (Mr 3,000) heparin fraction (□), hexasaccharide (■), tetrasaccharide (○), and disaccharide (●) are shown. The disaccharide is a single structural species (ΔUA2Sα1-4GlcNS6S). Assay details are described in Materials and Methods. Data shown are the mean and range of optical density measurements made in duplicate wells from a single experiment corrected for background signal, representative of three to four separate experiments. Mean IC_{50} values ± SD (μg/mL) from all experiments were: L-selectin-Ig: LMW, 28 ± 9; Hexa, 115 ± 12; Tetra, 240 ± 50; P-selectin-Ig: LMW, 105 ± 20; Hexa, 496 ± 57; Tetra, 1,019 ± 133.

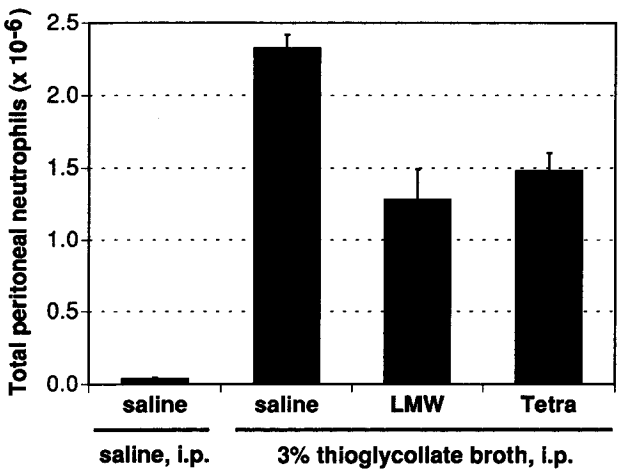


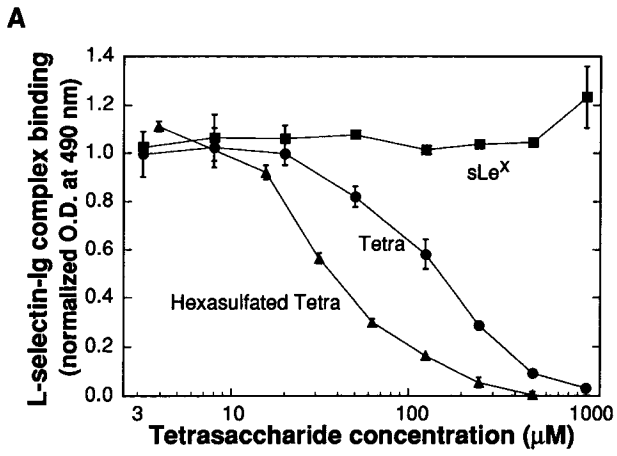
Fig 3. Low molecular weight (LMW) heparin and heparin-derived tetrasaccharides inhibit acute inflammation in vivo. Experimental groups (IP thioglycollate) contained five to seven animals per treatment in each experiment; control groups (IP saline) contained one to three animals in each experiment and were consistent with controls established by a large number of previous experiments. Intravenous injections of saline or saline containing 1.5 mg LMW heparin or heparin tetrasaccharides were administered 10 minutes after IP injections; peritoneal lavage was performed 120 minutes after IP injections. The graph shows the mean ± SEM of combined data from three separate experiments. Blocking by LMW heparin and heparin tetrasaccharide was statistically significant at $P < .001$ in a Student's t-test.

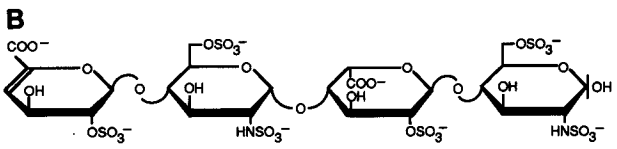
RESULTS AND DISCUSSION

In a competition ELISA, crude heparin inhibited the binding of L- and P-selectin-Ig, but not E-selectin-Ig, to immobilized BSA-sLe^x (Table 1). Heparan sulfate and de-N-sulfated heparin were also inhibitory, but required higher concentrations for equivalent activity (Table 1). Blocking activities of LMW heparin (a size-fractionated preparation averaging 10 monosaccharides) and of heparin-derived oligosaccharides are shown in Fig 1. The activities of size-defined oligosaccharides prepared by enzymatic cleavage of heparin were correlated to their length. Of particular note, heparin oligosaccharides with as few as four sugar residues inhibited both L- and P-selectin-Ig binding to BSA-sLe^x (Fig 1, A and B). Heparin hexasaccharides were approximately twofold more active than the tetrasaccharides. Heparin octasaccharides and decasaccharides showed increasing blocking activity approaching that of LMW heparin (not shown). In contrast, these oligosaccharides and LMW heparin failed to block E-selectin-Ig binding at concentrations up to 1 mg/mL (Fig 1C). This differential inhibition of the selectins by heparin corresponded to differential binding: L- and P-selectin-Ig bound to immobilized heparin-BSA (ELISA) and to heparin-Sepharose (column chromatography), whereas E-selectin-Ig binding to these substrates was not detected (data not shown).

To measure the ability of heparin-derived oligosaccharides to inhibit cell adhesion involving selectins, two assay systems were used. In the first, inhibition of LS180 colon cancer cell adhesion to immobilized selectin-Ig was measured. As depicted in Fig 2A, heparin molecules containing four or more monosaccharide residues at a concentration of 1 mg/mL inhibited LS180 adhesion to L- and P-selectin-Ig, but not to E-selectin-Ig. In a second assay system, COS cells were transfected with cDNAs encoding full-length transmembrane forms of each selectin and their ability to support adhesion of isolated human neutrophils was assessed. Few neutrophils bound to L-selectin transfected COS cells, whereas numerous rosettes (three or more neutrophils bound per COS cell) formed on P- and E-selectin transfected COS cell monolayers (Fig 2B). At a concentration of 1 mg/mL, heparin molecules containing four or more sugar residues inhibited this rosette formation on P-COS but not on E-COS. Similar results were obtained using the promyelocytic cell line HL60 (data not shown).

Although heparin has been used as an anticoagulant for more than 50 years, it can modulate a variety of biologic processes apart from hemostasis. For example, high molecular weight heparin affects immune responsiveness and inflammation in several animal models.[17-19] Recent reports demonstrate that crude heparin can inhibit leukocyte rolling on the vessel wall, a process known to involve the





ΔUA2S(1-4)-α-GlcNS6S(1-4)-α-IdoA2S(1-4)-α-GlcNS6S

Fig 4. (A) Comparison of the hexasulfated heparin tetrasaccharide (▲; ΔUA2Sα1-4GlcNS6Sα1-4IdoA2Sα1-4GlcNS6S), size-defined heparin tetrasaccharides (●), and the sLe^x tetrasaccharide (■; Neu5Acα2-3Galβ1-4[Fucα1-3]GlcNAc) as inhibitors of L-selectin-Ig binding to BSA-sLe^x in a competition ELISA. Data shown are the mean and range of normalized optical density measurements made in duplicate wells in separate representative experiments for each tetrasaccharide preparation. IC_{50} values calculated from combined experiments are: Tetra (heparin tetrasaccharides), 200 ± 41 μmol/L (mean ± SD, four experiments); Hexasulfated tetrasaccharide, 46 ± 5 μmol/L (mean ± range/2, two experiments). (B) Haworth projection of hexasulfated heparin tetrasaccharide.

selectins.[36,37] To study the effects of defined heparin oligosaccharides in vivo, we selected a murine model of thioglycollate-induced peritoneal inflammation that has been shown to involve both L- and P-selectin during initial neutrophil recruitment.[38-40] As shown in Fig 3, heparin tetrasaccharides administered intravenously diminished the number of neutrophils recovered by peritoneal lavage 2 hours after thioglycollate injection. These tetrasaccharides have no significant anticoagulant activity.[41] Moreover, intravenous administration of heparin tetrasaccharides did not significantly alter the number of neutrophils in the peripheral blood (not shown). LMW heparin had similar anti-inflammatory activity.

Heparin can bind to a diverse array of proteins. Some of these interactions may result chiefly from heparin's high density of negative charges imparted by its numerous carboxyl and sulfate groups. As might be expected, basic proteins, including histones, protamine, and histidine-rich glycoprotein, bind to heparin and to related acidic glycosaminoglycans such as heparan sulfate, chondroitin sulfate, and dermatan sulfate. However, the high-affinity binding of heparin to antithrombin requires a distinct sequence of five saccharides with the correct constellation of N- and O-sulfate groups.[6] Initial characterization of the structural requirements for heparin tetrasaccharide binding to selectins showed that a single hexasulfated tetrasaccharide, ΔUA2Sα1-4GlcNS6Sα1-4IdoA2Sα1-4GlcNS6S (Fig 4), is a particularly active competitive inhibitor. In the competition ELISA using L-selectin-Ig, this hexasulfated tetrasaccharide molecule was approximately fourfold more active than the heparin tetrasaccharide mixture (Fig 4), which contains this hexasulfated molecule along with other tetrasaccharides that have fewer sulfate moieties. In addition, the hexasulfated tetrasaccharide was found to be a more effective blocker of L-selectin-Ig binding to BSA-sLe^x than two pentasulfated heparin tetrasaccharides, ΔUA2Sα1-4GlcNS6Sα1-4IdoA2Sα1-4GlcN6S and ΔUA2Sα1-4GlcNS6Sβ1-4GlcAα1-4GlcNS6S (data not shown). As also shown in Fig 4, the sLe^x tetrasaccharide had no measurable blocking activity against L-selectin-Ig at concentrations up to 1 mmol/L. sLe^x has been reported to block approximately 50% of L-selectin-Ig binding to immobilized sLe^x glycolipid at a concentration of 5 mmol/L,[35] and approximately 60% of L-selectin-Ig binding to a high endothelial venule-derived glycoprotein, glycam-1, at 11 mmol/L.[42] Thus, heparin tetrasaccharides, and particularly the hexasulfated tetrasaccharide, are far more active inhibitors of L-selectin-Ig in these noncellular assays than is the sLe^x tetrasaccharide. By contrast, sLe^x inhibited E-selectin-Ig binding to immobilized BSA-sLe^x with an IC_{50} of 510 ± 60 μmol/L, whereas none of the heparins inhibited E-selectin-Ig binding.

Mounting evidence suggests that competitors of specific selectin ligands can diminish the inflammatory response. In a cobra venom factor-induced rat model of inflammation, for example, intravenously administered sLe^x-containing structures decreased lung permeability, hemorrhage, and myeloperoxidase content.[43] In another study, L-selectin-Ig diminished thioglycollate-induced neutrophil migration into the peritoneal cavities of mice.[38] Our data indicate that small, non-anticoagulant heparin molecules can block neutrophil accumulation during acute inflammation, and suggest that this activity depends, at least in part, on the ability of these oligosaccharides to block L- and P-selectin. Interestingly, heparin also binds to other participants in the inflammatory response, including complement components and certain cytokines. Because of the complexity of inflammatory disease processes, heparin's potential to interrupt diverse cellular events makes it an interesting candidate for further study as an anti-inflammatory therapeutic. Moreover, the demonstration of differential recognition of heparin oligosaccharides and sLe^x-like structures by the three selectins suggests that anti-inflammatory therapeutic reagents based on these two distinct classes of carbohydrates may act in a complementary manner.

ACKNOWLEDGMENT

We thank Dr R. MacGillivray for providing pNUT expression vector and BHK cells, Dr T. Tedder for providing anti–L-selectin monoclonal antibody LAM1.3, Dr R. McEver for anti–P-selectin monoclonal antibody G1, and Chembiomed LTD for BSA-sLe^x. We are grateful to D. Santoro, J. Collier, and D. Pursell for technical assistance, and D. Truell for manuscript preparation.

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