Inhibition of Selectin-mediated Cell Adhesion and Prevention of Acute Inflammation by Nonanticoagulant Sulfated Saccharides

STUDIES WITH CARBOXYL-REDUCED AND SULFATED HEPARIN AND WITH TRESTATIN A SULFATE*

Received for publication, February 14, 2000, and in revised form, July 24, 2000
Published, JBC Papers in Press, August 15, 2000, DOI 10.1074/jbc.M001257200

Xun Xie‡§, Anne-Sophie Rivier‡§, Andreas Zakrzewicz§, Michael Bernimoulin‡, Xian-Lu Zeng‡, Hans Peter Wessel‡, Marc Schapira‡, and Olivier Spertini‡**

From the Division and Central Laboratory of Hematology, Centre Hospitalier Universitaire Vaudois, Bugnon 46, 1011 Lausanne, Switzerland, the Institute of Physiology, Freie Universität, Berlin, Arminlaalee 22, 14195 Berlin, Germany, and the Pharmaceutical Research Department, Building 15/30F, Hoffmann-La Roche Ltd., 4002 Basel, Switzerland

Selectins play a major role in the inflammatory reaction by initiating neutrophil attachment to activated vascular endothelium. Some heparin preparations can interact with L- and P-selectin; however, the determinants required for inhibiting selectin-mediated cell adhesion have not yet been characterized. We now report that carboxyl-reduced and sulfated heparin (prepared by chemical modifications of porcine intestinal mucosal heparin leading to the replacement of carboxylates by O-sulfate groups) and trestatin A sulfate (obtained by sulfation of trestatin A, a non-uronic pseudo-nonasaccharide extracted from Streptomyces dimorphogenes) exhibit strong anti-P-selectin and anti-L-selectin activity while lacking antithrombin-mediated anticoagulant activity. In vitro experiments revealed that both compounds inhibited P-selectin- and L-selectin-mediated cell adhesion under laminar flow conditions. Moreover, carboxyl-reduced and sulfated heparin and trestatin A sulfate were also active in vivo, as assessed by experiments showing that 1) microinfusion of trestatin A sulfate reduced by 96% leukocyte rolling along rat mesenteric postcapillary venules and 2) that both compounds inhibited (by 58–81%) neutrophil migration into thioglycollate-inflamed peritoneum of BALB/c mice. These results indicate that nonanticoagulant sulfated saccharides targeted at P-selectin and L-selectin may have therapeutic potential in inflammatory disorders.

Leukocyte migration in inflammatory lesions is a reaction that is sequentially regulated by adhesion receptors and inflammation products. Selectins play a major role in initiating neutrophil attachment to cytokine-activated endothelium. L-selectin is expressed by most circulating leukocytes; E-selectin expression is induced after several hours of endothelial cell activation by interleukin-1, tumor necrosis factor-α, or endothelin; P-selectin is rapidly expressed by endothelial cells or platelets exposed to thrombin or histamine (1–6).

Due to different kinetics of expression, the various selectins function at different, although overlapping, phases of the inflammatory reaction. The earliest phase (<20 min) of neutrophil spontaneous rolling in rat postcapillary venules is mainly dependent on interactions between P-selectin and its major ligand P-selectin glycoprotein-1 (PSGL-1), whereas L-selectin involvement is observed during a later phase (7–12). Several observations indicated that PSGL-1 is a common ligand for L-, P-, and E-selectin (13–16). This mucin-like glycoprotein is expressed by most leukocytes and interacts with L-selectin and P-selectin through a N-terminal tyrosine sulfation consensus and sialylated, fucosylated core-2 branched O-glycans (4, 14, 17–24). Importantly, interactions between L-selectin and PSGL-1 mediate the attachment of circulating neutrophils to neutrophils already adherent to the endothelium, a process that may increase neutrophil recruitment in inflamed tissues (25, 26).

Selectins share a common primary structure with a N-terminal lectin domain that interacts with various glycoconjugated ligands. Most biological ligands of selectins are mucin-like glycoproteins. Four L-selectin ligands have been identified on high endothelial venule of peripheral lymph nodes, including CD34, GlyCAM-1, podocalyxin-like protein, and Sgp200 (27–30). These ligands present core-2 branched O-glycans terminated by sulfated isomers of sialyl Lewisα (SLeα) tetrasaccharides that are essential for binding to L-selectin (31–33). L-selectin ligands in inflamed microvascular venular or arterial endothelium have not yet been all identified. Sialylated and fucosylated ligands as well as heparan sulfate proteoglycans may play a role in this latter reaction (9, 34–44).

Several studies have indicated that heparan sulfate and heparin are ligands for L-selectin and P-selectin. Heparan sulfate proteoglycans isolated from calf pulmonary endothelial cells or kidney can interact with L-selectin (40–42, 45, 46). Moreover, heparin or heparin-like oligosaccharides can inhibit L-selectin or P-selectin binding to SLeα-related compounds or HL-60 cells (46, 47). Heparin is a glycosaminoglycan composed of alternating α-glucosamine and uronic acid (L-iduronic or D-glucuronic acid) residues that are heterogeneous in size and degree of sulfation. The heparin anticoagulant effect is caused by antithrombin activation, a reaction that follows a conformational change that takes place when the serpin binds to a specific pentasaccharide sequence. In contrast, little information is available on the structural determinants required for

* This work was supported by Grants 32-50632.97 and 32-54069.98 from the Swiss National Foundation for Scientific Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ These authors contributed equally to this study and share first authorship.

§ These authors contributed equally to this study and share first authorship.

** To whom correspondence should be addressed: Div. of Hematology, University of Lausanne, BH 18–543, 1011 CHUV Lausanne, Switzerland. E-mail: olivier.spertini@chuv.hospvd.ch.

The abbreviations used are: PSGL-1, P-selectin glycoprotein-1; mAb, monoclonal antibody; CHO, Chinese hamster ovary; CR, carboxyl-reduced; CRS, carboxyl-reduced and sulfated; UFH, unfractionated; LMWH, low molecular weight heparin; Fuc-T VII, fucosyltransferase VII; C2GnT, core-2 β1,6N-acetylgalactosaminyltransferase transferase.
L-selectin and P-selectin binding. The beneficial effects of selectin inhibitors in animal models of ischemia/reperfusion injury (for example in models of myocardial infarction, stroke, traumatic shock or solid organ transplantation) suggest that heparin or heparin-like compounds could be helpful in inhibiting selectin function and preventing tissue damage (48–54). However, heparin anticoagulant properties and the potential of bleeding complications may contraindicate its use as an anti-adhesive compound. In this study, we have examined whether the antithrombin-mediated anticoagulant activity of heparin could be separated from its anti-selectin activity. Two compounds with high anti-L-selectin and anti-P-selectin activity but negligible anticoagulant activity were identified including a chemically modified (carboxyl-reduced and sulfated) form of heparin and the non-uronic pseudo-nonasaccharide trestatin A sulfate. Furthermore, the anti-selectin activities of these compounds were compared with those of three unfractonated (UFH) and three low molecular weight heparin (LMWH) preparations approved for anticoagulant therapy in clinical practice.

EXPERIMENTAL PROCEDURES

Antibodies and Chimeric Selectins—The anti-L-selectin monoclonal antibodies (mAbs) LAM1–3, -10, and -11 (55); the anti-P-selectin mAb WASP12.2 (ATCC no. HB-299); and the anti-E-selectin mAb 7A9 (ATCC no. HB-10155) were purified from hybridoma culture supernatants using the MAPP II kit (Bio-Rad Laboratories, Glattbrugg, Switzerland). Anti-E-selectin mAb H18/7 (Fab’2 fragment) was a gift from F. W. Luscinskas (Brigham and Women’s Hospital, Harvard Medical School, Boston, MA). G1 mAb was a gift from R. P. McEver (Warren Medical Research Institute, University of Oklahoma Health Sciences Center, Oklahoma City, OK) or purchased from Bender MedSystem (Vienna, Austria). FLI mAb was purchased from Immunotech (Marcellin, France). L-selectin/Fc chimera (a, E-selectin/Fc, P-selectin/Fc, or CD4/µ chimeras) were produced by transient transfection of COS-7 cells using the DEAE method (14). After concentration of COS-7 cell culture medium, chimeric molecules were used for immunophenotypic studies.

Heparin and Oligosaccharide Preparations—The modified heparins and sulfated oligosaccharides used in this study showed nonparallel dose-response curves with the International Standard for Heparin. Therefore, the anticoagulant activities of these compounds were not expressed in IU of heparin, but characterized by the IC50 indicating the concentration of the compound leading to a clotting time of twice the control (56). Three heparin preparations were obtained from Hoffmann-La Roche. 1) The heparin (Fig. 1a) used as “control heparin” was heparin from Hoffmann-La Roche and had a mean molecular weight of 12,500, a sulfur content of 10.7%, an anti-IIa anticoagulant activity of 2.2 mg/ml, an anti-Xa activity of 2.7 µg/ml, and an IC50 on smooth muscle cell proliferation of 100 µg/ml (56). 2) CR-heparin (CR for carboxyl-reduced, Fig. 1a) was prepared by reduction of control heparin (56). During the reaction no depolymerization had occurred, as evidenced by the high performance liquid chromatography profiles on an Ultrapac TSK G 3000 gel permeation column in comparison with the starting material, as described previously (56, 57). CR-heparin had no detectable anti-IIa and anti-Xa anticoagulant activity and no inhibitory effect on smooth muscle cell proliferation. The sulfur content of CR-heparin was 12.2% (56). 3) CRS-heparin (CRS for carboxyl-reduced and sulfated; Fig. 1a) was obtained by sulfation of the primary hydroxyl groups of CR-heparin; the experimental procedure and characterization have been reported previously (56). CRS-heparin had a sulfur content of 15.7% and a low anticoagulant activity (anti-IIa activity: 170 mg/ml; anti-Xa activity: 680 µg/ml) (56). The selective sulfation of the primary hydroxyl groups was supported by characteristic peaks observed in the 13C NMR spectrum. The inhibitory effect of CRS-heparin on smooth muscle cell proliferation was higher than for the control heparin (relative inhibition (r) = 1.2) (58).

The following oligosaccharides were also obtained from Hoffmann-La Roche. Trestatin A, a non-uronic pseudo-nonasaccharide (Fig. 1b) extracted from Streptomyces dimorphogenes, had neither anticoagulant nor antiproliferative activity (56). Trestatin A sulfate (Maw = 3550 ± 300) was prepared by extensive sulfation of trestatin A (Maw = 1435), as previously reported (56). Briefly, trestatin A was dissolved in absolute N,N-dimethylformamide and sulfated in the presence of sulfur trioxide pyridine complex using excess of reagent (2.3 eq/hydroyxl group in trestatin A). After precipitation of the sulfated compound, the crude material was treated with aqueous sodium acetate solution to obtain the trestatin A sulfate sodium salt. A mixture of differently sulfated pseudo-nonasaccharides was obtained with an average number of sulfated groups per monosaccharide unit (degree of sulfation = 2.3 ± 0.3). The degree of sulfation for trestatin A sulfate was calculated from the integrals in the NMR spectrum of the pyridinium salt (56). Trestatin A sulfate had no anticoagulant activity as assessed by the activated partial thromboplastin time assay, and anti-IIa and anti-Xa activity in chromogenic assays, but a high inhibitory activity on smooth muscle cell proliferation (r = 1.2) (58).

Six commercially available heparin preparations, derived from porcine intestinal mucosa, were evaluated including three UFH and three LMWH. The following UFH were tested: UFH A, Liquemin® (Roche Ltd, Reinhach, Switzerland; lot B30MF111997); UFH B, heparin Novo® (Novo Industrie, Denmark; lot A470051); and UFH C, heparin Leo® (Leo Pharmaceutical, Ballerup, Denmark; lot C3750F). LMWH were: dalteparin (Low-Liquemin®, Roche Ltd, Reinach, Switzerland; lot B 20765B51), nadroparin (Fraxiparine®, Sanofi Winthrop, Münchenstein, Switzerland; lot 255) and enoxaparin (Clexane®, Rhône-Poulenc Rorer, Thalwil, Switzerland; lot 3813).

Cell Samples—Heparinized blood was obtained from healthy blood donors. Peripheral blood mononuclear cells were prepared by centrifugation on a Ficoll-Hypaque. Neutrophils were isolated from Ficoll-Hypaque pellets by dextran sedimentation followed by erythrocyte hypotonic lysis with 0.2% NaCl. CHO/dfr− cells were stably transfected with E-selectin or P-selectin cDNA subcloned in pCDNA3.1 vector (Invitrogen, Groningen, Switzerland) or with pcDNA3.1 vector alone, using LipofectAMINE (Life Technologies, Inc., Basel, Switzerland) and the procedure described by the manufacturer. Recombinant PSGL-1 was expressed in a glycosylated and functionally active form by CHO cells cotransfected with PSGL-1, core-2 β1,6N-acetylglucosaminyltransferase transfection vector (C2GnT), and fucosyltransferase VII (Fuc-T VII) cDNAs (13, 59, 60). PSGL-1 cDNA was subcloned in pCDNA3.1 vector; C2GnT and Fuc-T VII cDNAs were subcloned in the pZeo SV2+ vector (Invitrogen) modified in a IRES bicistronic expression vector. Transfected CHO cells were cultured in α-minimal essential medium (Life Technologies, Inc.) supplemented with ribonucleic acids, 0.4 mg/ml Geneticin (Life Technologies, Inc.), and 10% FCS (Life Technologies, Inc.).

Immunofluorescence Studies—Immunostaining was carried out by incubating cells for 20 min at 4°C with appropriate fluorescein isothiocyanate/phycocerythrin-conjugated mAbs or soluble adhesion receptors (CD18, CD11b; P-selectin; anti-L-selectin, anti-P-selectin, or CD4/µ chimeras) (14). mAbs and chimeric proteins were used at optimal concentrations in phosphate-buffered saline supplemented with 1% albumin and 1 mM CaCl2. Cell surface binding of chimeric proteins was detected using a polyclonal fluorescein isothiocyanate-conjugated rabbit anti-human IgM heavy chain antibody (Dako, Glostrup, Denmark). Flow cytometry was performed with a EPICS Profile cytofluorimeter (Coulter Electronic, Hialeah, FL). Mononuclear cells were gated using forward and right side scatter signals. A total of 5000 cells was analyzed in each experiment.

Cell Adhesion Assays—A well defined laminar flow was produced over confluent CHO cells stably expressing PSGL-1/C2GnT and Fuc-T VII (CHO-PSGL-1/C2GnT/Fuc-T VII cells; e-selectin (CHO-E-selectin cells), or P-selectin (CHO-P-selectin cells). Cells were grown on 25-mm glass circular coverslips introduced in a parallel plate flow chamber (40). Peripheral blood neutrophils or U937 cells, suspended at 0.5 × 106/ml in RPMI medium plus 1% FCS, were perfused through the chamber via a syringe pump (Harvard Apparatus, Indulab AG, Switzerland) for 4 min at room temperature and at a constant shear stress of 1.5 dyn/cm2. Neutrophil interactions with transfected CHO cells were visualized using an inverted phase contrast videomicroscope (Leica, Lausanne, Switzerland) and Sony CCD-IRIS video cameras) and video taped (Panasonic s-VHS recorder, TSA Telecom, Switzerland). Sequential images of neutrophil or U937 cell interactions with transfected CHO cells were digitalized and analyzed using a software developed for use in the public domain (National Institutes of Health Image software, version 1.57). Images were analyzed on a Power-Macintosh 8600/200 equipped with a Scion LG-3 board (Scion, Frederick, MD). Monolayer cell monolayers were analyzed from flow chamber at 2–4 min of perfusion. Most U937 cells interacting with CHO-P-selectin cells and most neutrophils interacting with CHO-PSGL-1/C2GnT/Fuc-T VII cells were rolling cells. Heparin preparations and oligosaccharides were diluted in cell suspensions at appropriate concentrations. The LAM 1–3, WASP12.2, and H18/7 or TAH mAbs were used as anti-L-selectin, anti-P-selectin, and anti-E-selectin function blocking mAbs. Isotype
matched mAbs were used as controls. Experiments were performed in quadruplicate. Results were expressed as percentage of the control obtained from experiments using cells in medium with vehicle alone. These control studies employing cells in medium with vehicle alone were conducted at the beginning and the end of each experimental condition.

**Intravital Microscopy**—Harlan Sprague-Dawley rats (250–300 g) were anesthetized with ketamine (75 mg/kg administered intramuscularly; Parke Davis, Berlin, Germany) after premedication with pentobarbital (Nembutal, Sanofi, Hannover, Germany; 20 mg/kg administered intramuscularly). Anesthesia was maintained by a continuous infusion of pentobarbital (0.2 mg/ml in physiologic saline) at 40 ml/kg/h. Leukocyte concentration was determined at 45-min intervals using a Coulter D<sub>3</sub> cell counter (Coulter, Herts, United Kingdom). After opening of the peritoneal cavity, a few loops of ileum proximal to the appendix were exteriorized onto a limited stage and were superfused with bicarbonate-buffered isotonic saline at 37 °C (61). Oligosaccharides or phosphate-buffered saline were infused through a micropipette in an upstream side branch of the venule to be investigated. Mesenteric microcirculation was observed using a Leitz (Wetzlar, Germany) intravital microscope and recorded on a Sony U-Matic videocassette (Sony, Berlin, Germany) via a video camera (RCA, Lancaster, PA). Rolling leukocyte flux was determined by counting the number of rolling leukocytes passing a line perpendicular to the vessel axis (61). For comparision between observations made in different venules, flux values were normalized to the average rolling leukocyte flux during the control period that followed each micro-infusion.

**Thioglycollate-induced Peritonitis**—BALB/c mice (6 weeks old, ~30 g) were anesthetized with metofane (Aroyet AG, Switzerland) and treated with a subcutaneous injection of 3.0 mg of tretastin A sulfate or CRS-heparin. Doses of CR-heparin, CRS-heparin, and tretastin A sulfate were in the same range as those chosen for thrombosis prophylaxis or for inhibition of L-selectin or P-selectin activity in mice (47). After 30 min, a point time that corresponds to the peaks blood levels of the injected drugs, the animals received an intraperitoneal injection of 1 ml of 3% thioglycollate broth (T9032, Sigma) or sterile pyrogen-free saline. The mice were sacrificed 3 h later, at a time point where both P-selectin and L-selectin are involved in regulating neutrophil migration into the peritoneal cavity (62, 63). Peritoneal leukocytes were harvested by peritoneal lavage with 5 ml of saline containing 2 mM EDTA. After red blood cell lysis, leukocytes were counted in a hemocytometer. Neutrophils were counted after staining with Türk or by counting cytospin preparations stained with Giemsa (Fluka, Switzerland).

**Statistical Analysis**—The Mann-Whitney test was used to compare medians of unpaired groups while medians of paired groups were compared with the Wilcoxon signed rank test. When three or more groups were compared, differences between treatments were evaluated by analysis of variance and Bonferroni multiple comparison tests. p values < 0.05 were considered as significant. Data are shown as means ± 1 S.E.

**RESULTS**

**Modulation of Selectin/µ Chimera Binding to KG-1 Cells or Peripheral Blood Neutrophils by Control Heparin, CR-heparin, or CRS-heparin**—Although several studies have shown that certain heparin preparations can inhibit L- or P-selectin-mediated interactions (41, 42, 46, 47), the structural determinants of heparin required for this inhibitory activity have not been identified. This issue was examined here using heparin preparations with well defined chemical modifications (Fig. 1a). The role of the carboxyl group of the uronic acid unit of heparin and of its replacement by a sulfate group was examined using competitive binding assays assessing L-selectin/µ, P-selectin/µ, or E-selectin/µ chimera binding to KG-1 cells and to neutrophils; this issue was also investigated with adhesion assays in a parallel plate flow chamber using laminar flow conditions. Control heparin, CR-heparin, or CRS-heparin differ in structure only at the level of the carboxyl group of the uronic acid unit of heparin. This carboxyl group is reduced in CR-heparin; the resulting hydroxyl group is sulfated in CRS-heparin (Fig. 1). In the selectin binding assay used here, L-selectin/µ, P-selectin/µ, or E-selectin/µ chimera binding to KG-1 cells or neutrophils was completely inhibited by 5 mM EDTA (Fig. 2, top panels, dotted lines) or specific adhesion blocking mAbs (anti-L-selectin mAb LAM1–3, anti-E-selectin mAb H18/7, anti-P-selectin mAb G1; data not shown) (14). The major part of L-selectin/µ and P-selectin/µ binding to KG-1 cells was inhibited by anti-FSGL-1 mAb PL1 or rabbit anti-FSGL-1 (42–56) polyclonal Ab raised against the first 15 amino acid residues of PACE-cleaved FSGL-1 (14). As illustrated in Fig. 2, control heparin (100 µg/ml) partially reduced L- or P-selectin/µ binding to KG-1 cells (p = 0.04, n = 3) and 20 ± 4% (n = 7, p = 0.02; Fig. 2). The same concentration (100 µg/ml) of CR-heparin did not significantly affect L-selectin/µ or P-selectin/µ binding to KG-1 cells (9 ± 2% (n = 3) and 6 ± 1% (n = 3)). In contrast, CRS-heparin (100 µg/ml) had high inhibitory activity against both L-selectin/µ and P-selectin/µ binding to KG-1 cells. In presence of CRS-heparin, L-selectin/µ...
binding to KG-1 cells was reduced by 38 ± 9% (p = 0.003, n = 4) and P-selectin/µ binding by 50 ± 6% (p = 0.0005, n = 7). However, neither control heparin, CR-heparin, nor CRS-heparin had noticeable effects on E-selectin/β cells and E-selectin/β (Fig. 2). In four experiments, E-selectin/µ binding to neutrophils was reduced by 0.4 ± 11% in the presence of control heparin (400 µg/ml), by 5 ± 16% in the presence of CR-heparin (400 µg/ml), and by 1 ± 5% in the presence of CRS-heparin (400 µg/ml) (Fig. 2).

These results show that the presence of carboxyl group in heparin is important for anti-L-selectin or anti-P-selectin activity. However, it can be artificially replaced by an O-sulfate ester, giving a compound with a more potent anti-L-selectin and anti-P-selectin activity without antithrombin-mediated anticoagulant activity.

Anti-L-selectin and Anti-P-selectin Activity of Sulfated Non-uronic Oligosaccharides—Previous observations indicated that carboxylates in heparin are not required to inhibit smooth muscle cell proliferation (56, 58), which had led to the investigation of the properties of series of non-uronic sulfated oligosaccharides. Among these oligosaccharides, trestatin A sulfate (Fig. 1b) was the most efficient at inhibiting the growth of smooth muscle cells (56). This sulfated oligosaccharide was therefore selected for assessment of its anti-selectin activity. Trestatin A is a pseudononasaccharide obtained from S. dimorphogenes (56). Trestatin A (1000 µg/ml) had no inhibitory effect on L-selectin/µ binding to KG-1 cells (−18 ± 9% of inhibition, n = 6; Fig. 3) nor did it affect P-selectin/µ binding to KG-1 cells (−4 ± 2%, n = 5; Fig. 3) and neutrophils (−3 ± 1%, n = 9; not illustrated) or E-selectin/µ binding to neutrophils (−1 ± 5%, n = 5; Fig. 3). In contrast, trestatin A sulfate (400 µg/ml) efficiently inhibited L-selectin/µ binding to KG-1 cells (70 ± 5% of inhibition, n = 6, p < 0.0001; Fig. 3). Trestatin A sulfate also inhibited P-selectin/µ binding to KG-1 cells (31 ± 6%, n = 5, p = 0.0008; Fig. 3) or neutrophils (43 ± 12%, n = 9, p = 0.006; not illustrated). Like CRS-heparin, trestatin A sulfate (800 µg/ml) did not affect E-selectin/µ binding to neutrophils (2 ± 7%, n = 5; Fig. 3).

Inhibition of L- and P-selectin-mediated Leukocyte Adhesion by Control Heparin, CR-heparin, CRS-heparin, and Trestatin A Sulfate: Studies under Shear Flow Conditions—The ability of control heparin, CR-heparin, CRS-heparin, or trestatin A sulfate to inhibit L-selectin- and P-selectin-mediated cell adhesion was then studied under flow conditions. Adhesion assays were performed in a parallel plate flow chamber, at a constant shear stress of 1.5 dyn/cm², on CHO cell monolayers stably expressing E-selectin or P-selectin at levels similar to those expressed by activated human endothelial cells. For studying interactions between P-selectin and PSGL-1, we used U937 cells expressing high levels of PSGL-1, C2GnT, and Fuc-T VII and CHO-P-selectin cell monolayers. For studying interactions between L-selectin and PSGL-1, we used peripheral blood neutrophils and CHO cell monolayers expressing high levels of PSGL-1, C2GnT, and Fuc-T VII. It was observed that U937 cells efficiently interacted with CHO-P-selectin cells (57 ± 9 rolling cells/min/m², n = 19). The specificity of this interaction was demonstrated by blocking studies with specific mAbs. WASP 12.2, an anti-P-selectin mAb, abolished the rolling of U937 cells on CHO-P-selectin cells (99 ± 1% of inhibition, n = 8). Conversely, anti-PL1 mAb eliminated PSGL-1-dependent interactions of U937 cells with CHO-P-selectin cells (99 ± 1% of inhibition, n = 4).

The anti-P-selectin activity of control heparin, CR-heparin, CRS-heparin, trestatin A, and trestatin A sulfate were initially determined at a saccharide concentration of 1.0 mg/ml. Control heparin strongly inhibited U937 cell rolling on CHO-P-selectin cells (96 ± 2% of inhibition, p < 0.001, n = 4; Fig. 4). Carboxyl reduction of control heparin caused a complete loss of this inhibitory activity. Indeed, rolling of U937 cells on P-selectin was not significantly inhibited by 1.0 mg/ml CR-heparin (21 ± 12% of inhibition, n = 4). However, sulfation of CR-heparin reestablished anti-P-selectin activity. For example, the rolling of U937 cells on CHO-P-selectin cells was almost abolished in presence of 1.0 mg/ml CRS-heparin (98 ± 1% of inhibition, p < 0.001, n = 8; Fig. 4). A similar inhibition of U937 cell rolling on CHO-P-selectin cells was seen with trestatin A sulfate (99 ± 1%, p < 0.001, n = 8; Fig. 4). No anti-P-selectin activity was detectable with 1.0 mg/ml trestatin A (84 ± 10%, n = 4; Fig. 4); emphasizing the importance of sulfate groups for observing antiadhesive activity. The anti-P-selectin activities of control heparin, CR-heparin, and trestatin A sulfate were then quantified by estimating IC₅₀ values for the inhibition of U937
Nonanticoagulant Sulfated Saccharides Inhibit L-/P-selectin

Neutrophils efficiently rolled on CHO-E-selectin cell monolayers (183 ± 8 rolling cells/mm²/min, n = 64). Neutrophil binding to CHO-E-selectin cells was abolished by cell treatment with the anti-E-selectin mAb 7A9 (0 ± 0 rolling cells/mm²/min, n = 8). At 1.0 mg/ml, the various saccharides under investigation did not inhibit U937 cell rolling on CHO-E-selectin cells (medium: 105 ± 20% (% of control, n = 12); control heparin: 110 ± 12% (n = 8); CR-heparin: 102 ± 13% (n = 4); CRS-heparin: 120 ± 10% (n = 12); trestatin A: 114 ± 14% (n = 4); trestatin A sulfate: 90 ± 6% (n = 8)).

Leukocyte Rolling Along Mesenteric Postcapillary Venules Is Inhibited by Trestatin A Sulfate—The effect of trestatin A sulfate on neutrophil rolling was then assessed in vivo. Microinfusions (26 experiments) were performed in mesenteric postcapillary venules of 8 rats. Blood leukocyte counts remained stable during the experimental protocol. Microinfusion of vehicle (phosphate-buffered saline) did not change rolling leukocyte flux. Microinfusion of trestatin A sulfate (1.0 mg/ml) inhibited rolling leukocyte flux by 96 ± 1% (seven applications, Fig. 7). The inhibitory effect was almost immediate, rolling leukocytes disappearing within 10 s of microinfusion (data not illustrated). The inhibitory effect of trestatin A sulfate was not sustained and it vanished within 10 s of termination of microinfusion. In keeping with in vitro observations, unsulfated trestatin A did not affect leukocyte rolling (Fig. 7).

CRS-heparin or Trestatin A Sulfate Inhibit Neutrophil Migration into Thioglycollate-inflamed Peritoneum—The ability of CRS-heparin and trestatin A sulfate to reduce neutrophil migration at sites of acute inflammation was evaluated in BALB/c mice using a thioglycollate-induced model of peritonitis. Three hours after thioglycollate injection, neutrophils were collected from the inflamed peritoneal cavity and counted. Thioglycollate injection induced a 4-fold increase in neutrophil accumulation. Neutrophil migration into the peritoneal cavity was efficiently inhibited by a subcutaneous injection of trestatin A sulfate (3.0 mg) or CRS-heparin (3.0 mg). In two experiments, neutrophil accumulation was reduced by 81 ± 11% (p < 0.001, n = 5) and 73 ± 10% (p < 0.001, n = 7, Fig. 8) when mice were treated with trestatin A sulfate, whereas the unsulfated form of this nonasaccharide had no effect. Subcutaneous injection of CRS-heparin inhibited neutrophil migration by 58 ± 10% (p < 0.01, n = 5) and 73 ± 10% (p < 0.01, n = 5, Fig. 8). As expected, CR-heparin (3.0 mg subcutaneous) did not influence neutrophil migration (Fig. 8).

Inhibition of L-selectin- and P-selectin-mediated Cell Adhesion by UFH and LMWH Anticoagulant Heparins—Three UFH and three LMWH preparations used in clinical practice for prophylaxis or treatment of venous thrombosis were studied for...
their anti-selectin activity. Selectin-mediated attachment was assessed by evaluating U937 cell rolling on CHO cells expressing P-selectin (P-selectin-dependent cell adhesion). The antiadhesive effect of UFH, diluted at 150 USP units/ml, was highly variable among three formulated pharmaceutical preparations, A, B, and C. UFH C inhibited U937 cell rolling by 98 ± 1% (p < 0.001, n = 8), whereas UFH A had no significant inhibitory effect (2 ± 21%, n = 6). UFH B had an intermediate effect and decreased rolling by 61 ± 8% (p < 0.001, n = 8). Three formulated LMWH were used at 1.0 mg/ml. Dalteparin (1.0 mg/ml, *i.e.* 158 anti-Xa units/ml) inhibited U937 rolling on CHO-P-selectin cells by 92 ± 3% (n = 8, p < 0.001). Enoxaparin had a weaker inhibitory effect; at a concentration of 1.0 mg/ml (100 anti-Xa units/ml), enoxaparin inhibited U937 cell rolling on P-selectin by 51 ± 12% (p = 0.01, n = 9). In contrast, nadroparin (1.0 mg/ml) did not significantly reduce U937 cell rolling on P-selectin (27 ± 7%; n = 6, p = 0.2). Of note none of the heparin preparations mentioned above inhibited L-selectin-dependent neutrophil rolling on PSGL-1.

**FIG. 8.** Effect of CR-heparin, CRS-heparin, trestatin A, and trestatin A sulfate on neutrophil migration in inflamed rat peritoneum. Each experimental group contained 5–7 BALB/c mice. Control mice were injected with sterile, pyrogen-free saline alone (*white column*); neutrophil migration 3 h after the injection of 3% thioglycollate broth (*black or hatched columns*) is shown. Saline and oligosaccharides were injected subcutaneously 30 min before thioglycollate. Results, indicated as mean ± S.E., are representative of two separate experiments (***, p < 0.01; ***, p < 0.001).
activity of CRS-heparin and trestatin A sulfate is a common feature of biological selectin ligands which regulate leukocyte migration (67, 68). Specific sulfotransferases are involved in the sulfation of sialyl-6-sulfo-Lea and sialyl-6-sulfo-Lea, two capping structures of GlyCAM-1 and CD34 that interact with L-selectin (32, 33, 68). Sulfation of PSGL-1 is regulated by two tyrosylprotein sulfotransferases and is required for L-selectin and P-selectin binding (14, 17, 18, 22, 70, 71). Sulfation of heparan sulfate proteoglycans may also be important to support L-selectin binding (40–42).

Since an intact uronic acid unit is not required for inhibiting L-selectin or P-selectin binding to PSGL-1, we examined the anti-selectin activity of trestatin A sulfate, a highly sulfated pseudo-nonaccharide with antiproliferative activity on arterial smooth muscle cells (56). Trestatin A sulfate exhibited high anti-L-selectin and anti-P-selectin activities (Figs. 4–6). Trestatin A sulfate was very efficient at inhibiting L-selectin-mediated rolling on PSGL-1, whereas CRS-heparin had a much weaker effect on this reaction and control heparin was completely inactive (Fig. 6). In vivo, the inhibitory activity of trestatin A sulfate on neutrophil rolling along postcapillary venules disappeared quickly upon termination of microinfusion. Transient inhibition of selectin-mediated rolling might be advantageous, as it will probably avoid prolonged suppression of the host cellular defense system. Importantly, sulfation of trestatin A was required for anti-L-selectin and anti-P-selectin activity, further emphasizing the role of sulfate residues for L-selectin and P-selectin binding to carbohydrate ligands (Fig. 7).

Three UFH and three LMWH had highly variable anti-P-selectin activity. UFH A, a preparation extracted from porcine intestinal mucosa, had no detectable anti-L-selectin and anti-P-selectin activity, whereas UFH C had a strong anti-P-selectin activity and UFH B a moderate inhibitory effect on P-selectin-mediated rolling. These observations indicate that the exhibition of an anti-selectin activity is not a general property of UFH, a notion consistent with the data available in the literature. In some studies, certain heparin preparations were found to have significant inhibitory activity against L-selectin or P-selectin; in other studies, selectin inhibition was absent and no reaction was observed with heparin or heparan sulfate proteoglycans (41, 46, 47, 72, 73). These differences suggest that certain heparin could express unique structural features that support L-selectin and P-selectin binding. Differences in molecular weight, degree of sulfation, as well as by the expression of specific binding sequences for L- or P-selectin may be important.

Carboxyl reduction and sulfation of heparin or sulfation of non-uronic oligosaccharides such as trestatin A generates non-anticoagulant compounds with high anti-L-selectin and anti-P-selectin activities. These compounds have the potential of preventing leukocyte migration with reduced bleeding risk. The anti-inflammatory activity of CRS-heparin and trestatin A sulfate was evaluated in a thiglycollate-induced model of peritonitis. Subcutaneous injection of trestatin A sulfate and CRS, before thiglycollate intraperitoneal injection, inhibited neutrophil migration by 60–80%, confirming the potent inhibitory activity of these compounds on neutrophil adhesion and migration (Fig. 8). Neutrophil migration in this peritonitis model is dependent on E-selectin, P-selectin, and L-selectin function (74–76). Neutrophil migration was not completely inhibited despite blockade of L-selectin and P-selectin by CRS-heparin or trestatin A sulfate. Residual migration may result from E-selectin-dependent cell adhesion, a reaction not affected by CRS-heparin or trestatin A sulfate. Inhibition of cell migration could be enhanced by combining E-selectin inhibitors to CRS-heparin or trestatin A sulfate (77, 78).

In clinical practice, inhibitors of L-selectin and P-selectin could be very useful to prevent ischemia/reperfusion injury observed in various conditions such as myocardial infarction, stroke, or solid organ transplantation. The role of L-selectin- and P-selectin-dependent cell adhesion in contributing to ischemia/reperfusion injury has been well established in animal models (48, 50–54, 79–82). Inhibition of L-selectin or P-selectin with mAbs or sLex-related compounds attenuates neutrophil accumulation in reper fused organ, reduces the area at risk of infarction or acute organ dysfunction, and results in better recovery. Some heparin preparations can preserve myocardial contractility after ischemia/reperfusion injury or reduce brain injury (83–86). The anti-L-selectin and anti-P-selectin activity of heparin could constitute a major mechanism by which heparin derivatives may prevent reperfusion injury. Additional properties of heparin could also contribute to reduce reperfusion injury such as inhibition of complement activation or CD11b function (69, 73, 87). The anti-L-selectin and anti-P-selectin activity of CRS-heparin and trestatin A sulfate could be particularly useful for the prevention of reperfusion injury when the risk of hemorrhagic complications is increased, for example in the case of thrombolytic therapy, traumatic shock, or solid organ transplantation. Additional in vivo studies are now required to further assess the therapeutic potential of these compounds.

Acknowledgments—We are grateful to Dr. Philippe Schneider, Dr. Jean-Daniel Tissot, and the staff of the Centre de Transfusion Sanguine (Lausanne, Switzerland) for providing blood samples. We thank Dr. Roger McEver for providing the anti-P-selectin mAb G1, Dr. F. W. Luscinakas for H18/7 mAb, Dr. J. Lowe for Fuc-T VII cDNA, and Dr. M. Fukuda for C2GNT cDNA.

REFERENCES
1. Kansas, G. S. (1996) Blood 88, 3259–3267
2. Springer, T. A. (1994) Cell 76, 301–314
3. Rosen, S. D., and Bertozzi, C. R. (1994) Curr. Opin. Cell Biol. 6, 663–673
4. McEver, R. P., and Cummings, R. D. (1997) J. Clin. Invest. 100, 485–491
5. McEver, R. P., Moore, K. L., and Cummings, R. D. (1995) J. Biol. Chem. 270, 11025–11028
6. Teder, T. F., Steeber, D. A., Chen, A., and Engel, P. (1995) FASEB J. 9, 866–873
7. Ley, K., Bullard, D. C., Arbones, M. L., Bosse, R., Restweber, D., Teder, T. F., and Beaudet, A. L. (1995) J. Exp. Med. 181, 669–675
8. Jung, U., Bullard, D. C., Teder, T. F., and Ley, K. (1996) Am. J. Physiol. 271, H2740–H2747
9. Ley, K., Teder, T. F., and Kansas, G. S. (1993) Blood 82, 1632–1638
10. Norman, K. E., Moore, K. L., McEver, R. P., and Ley, K. (1996) Blood 88, 4417–4421
11. Davenpeck, K. L., Steeber, D. A., Teder, T. F., and Bochner, B. S. (1997) J. Immunol. 158, 1577–1586
12. Yang, J., Hirata, T., Chen, A. J., Delahunty, M. D., Moore, K. L., Watson, S. R., McEver, R. P., and Cummings, R. D. (1999) Am. J. Pathol. 211, 1769–1782
13. Sako, D., Chang, X.-J., Barone, K. M., Vachino, G., White, H. M., Shaw, G., Veldman, G. M., Bean, K. M., Aberr, T. J., Furie, B., Cumming, D. A., and Larsen, G. R. (1993) Cell 75, 1179–1186
14. Sperandio, O., Cordery, A.-S., Monat, N., Guißler, F., and Schapira, M. (1996) J. Cell Biol. 135, 523–531
15. Walcheck, B., Moore, K. L., Meever, R. P., and Kishimoto, T. K. (1996) J. Clin. Invest 98, 1081–1087
16. Tu, L. L., Chen, A. J., Delahunty, M. D., Moore, K. L., Watson, S. R., McEver, R. P., and Teder, T. F. (1996) J. Immunol. 157, 3995–4004
17. Pouyani, T., and Seed, B. (1995) Cell 83, 333–343
18. Norman, K. E., Moore, K. L., McEver, R. P., and Ley, K. (1996) Blood 88, 3255–3264
19. Wilkins, P. P., Moore, K. L., McEver, R. P., and Cummings, R. D. (1995) J. Biol. Chem. 270, 22677–22680
20. Moore, K. L., Eaton, S. F., Lyons, D., Lichenstein, H. S., Cummings, R. D., and McEver, R. P. (1994) J. Biol. Chem. 269, 23118–23127
21. Li, P., Wilkins, P. P., Crowley, S., Weinstein, J., Cummings, R. D., and McEver, R. P. (1996) J. Biol. Chem. 271, 3235–3242
22. Liu, W., Ramachandran, V., Kang, J., Kishimoto, T. K., Cummings, R. D., and McEver, R. P. (1998) J. Biol. Chem. 273, 7078–7085
23. Ramachandran, V., Nollett, M. U., Qiu, H. Y., Liu, W. J., Cummings, R. D., Zhu, C., and McEver, R. P. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 3771–3776
24. Leppanen, A., Mehta, P., Ouyang, Y. B., Ju, T. Z., Helin, J., Moore, K. L., van Die, I., Canfield, W. M., McEver, R. P., and Cummings, R. D. (1999) J. Biol. Chem. 274, 24388–24348
25. Bargatze, R. F., Kurk, S., Butler, E. C., and Jutila, M. A. (1994) J. Exp. Med. 180, 1785–1792
