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Abstract

Heparin is an excellent inhibitor of P- and L-selectin binding to the carbohydrate determinant, sialyl Lewis(x). As a consequence of its anti-selectin activity, heparin attenuates metastasis and inflammation. Here we show that fucosylated chondroitin sulfate (FucCS), a polysaccharide isolated from sea cucumber composed of a chondroitin sulfate backbone substituted at the 3-position of the beta-D-glucuronic acid residues with 2,4-disulfated alpha-L-fucopyranosyl branches, is a potent inhibitor of P- and L-selectin binding to immobilized sialyl Lewis(x) and LS180 carcinoma cell attachment to immobilized P- and L-selectins. Inhibition occurs in a concentration-dependent manner. Furthermore, FucCS was 4-8-fold more potent than heparin in the inhibition of the P- and L-selectin-sialyl Lewis(x) interactions. No inhibition of E-selectin was observed. FucCS also inhibited lung colonization by adenocarcinoma MC-38 cells in an experimental metastasis model in mice, as well as neutrophil recruitment in two models of inflammation (thioglycollate-induced peritonitis and lipopolysaccharide-induced lung inflammation). Inhibition occurred at a dose that produces no significant change in plasma activated partial thromboplastin time. Removal of the sulfated fucose branches on the FucCS abolished the inhibitory effect in vitro and in vivo. Overall, the results suggest that invertebrate FucCS may be a potential alternative to heparin for blocking metastasis and inflammatory reactions without the undesirable side effects of anticoagulant heparin.
SELECTIN-BLOCKING ACTIVITY OF A FUCOSYLATED CHONDROITIN SULFATE GLYCOSAMINOGLYCAN FROM SEA-CUCUMBER: EFFECT ON TUMOR METASTASIS AND NEUTROPHIL RECRUITMENT*

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Running title: Anti-selectin activity of fucosylated chondroitin sulfate
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Heparin is an excellent inhibitor of P- and L-selectin binding to the carbohydrate determinant, sialyl Lewisα. As a consequence of its anti-selectin activity, heparin attenuates metastasis and inflammation. Here we show that fucosylated chondroitin sulfate (FucCS), a polysaccharide isolated from sea cucumber composed of a chondroitin sulfate backbone substituted at the 3-position of the β-D-glucuronic acid residues with 2,4-disulfated α-L-fucopyranosyl branches, is a potent inhibitor of P- and L-selectin binding to immobilized sialyl Lewisα and LS180 carcinoma cell attachment to immobilized P- and L-selectins. Inhibition occurs in a concentration-dependent manner. Furthermore, FucCS was 4-8 folds more potent than heparin in the inhibition of the P- and L-selectin-sialyl Lewisα interactions. No inhibition of E-selectin was observed. FucCS also inhibited lung colonization by adenocarcinoma MC-38 cells in an experimental metastasis model in mice, as well as neutrophil recruitment in two models of inflammation (thioglycollate-induced peritonitis and LPS-induced lung inflammation). Inhibition occurred at a dose that produces no significant change in plasma aPTT. Removal of the sulfated fucose branches on the FucCS, abolished the inhibitory effect in vitro and in vivo. Overall, the results suggest that invertebrate FucCS may be a potential alternative to heparin for blocking metastasis and inflammatory reactions without the undesirable side effects of anticoagulant heparin.

The surface of carcinoma cells exhibits altered glycosylation patterns (1-5), often containing highly branched or sialylated oligosaccharides, especially fucosylated glycans such as sialyl-LewisX (Siaα2-3Galβ1-4(Fucα1-3)GlcNAc) and sialyl-LewisA (Siaα2-3Galβ1-3(Fucα1-4)GlcNAc). The presence of these oligosaccharides in tumor cells directly correlates with a poor prognosis for cancer patients because of tumor progression and metastatic spread (1-5). The sLeX-oligosaccharides from carcinoma cells act as ligands of the 3 members of the selectin family of cell adhesion molecules. E-, P- and L-selectins are vascular receptors for certain normal glycoproteins that contain sialyl-LewisA and found on leukocytes and endothelium (6-8). The selectins
also participate in hematogenous metastasis by mediating the interactions of tumor cells with platelets and endothelium (1-3). Hematogenous metastasis occurs through a series of sequential events involving the extravasation of tumor cells into the bloodstream, evasion of innate immune surveillance, adhesion to vascular endothelium of distant organs with subsequent extravasation and colonization of tissues. It has been proposed that these microemboli of tumor cells with platelets and leukocytes allows tumor cells to evade the immune defenses and eventually colonize distant organs, forming metastatic foci (9-15). Several studies have shown that a few minutes after intravenous injection, tumor cells are detected in emboli inside pulmonary capillaries in association with platelets and fibrin.

Studies from several groups have indicated that tumor metastasis in experimental animals is inhibited by heparin (16-19). Some clinical studies have also shown a beneficial effect of heparin in some types of human cancer (20-24). The antimitastatic effect of heparin does not reflect its anticoagulant activity (25,26), but rather relates to heparin’s ability to inhibit the interaction of sialyl Lewisxα-rich oligosaccharides on tumor cells with P-selectin on platelets (16,27). In the presence of heparin, tumor cells lose the protection conferred by platelets becoming susceptible to the potentially cytotoxic action of immune effector cells, which leads to the inhibition of metastasis. A single intravascular injection of heparin promotes immediate attenuation of the interaction of tumor cells with platelets, resulting in a marked reduction of metastasis (16).

The recruitment of leukocytes from blood to inflamed or injured tissues is also facilitated by E-, P- and L-selectins that mediate the initial rolling events on activated endothelium (28-29). Experiments in vivo show that heparin has a potent anti-inflammatory activity, blocking P- and L-selectins (30). Structural studies indicate that the inhibition of L- and P-selectin by heparin requires the presence of 6-O-sulfated glucosamine residues in the heparin molecule (30). Subsequent studies showed a similar requirement for blocking experimental metastasis (31-32).

Previously, we isolated a polysaccharide from the body wall of the sea cucumber *Ludwigothurea grisea*, which has a backbone like mammalian chondroitin sulfate: [4-β-D-GlcA-1→3-β-D-GalNAc]n, mostly 6-sulfated, but substituted at the 3-O-position of the glucuronic acid residues with 2,4 disulfated α-L-fucopyranose branches (FucCS) (33,34). The sulfated α-L-fucopyranose branches can be removed by mild acid hydrolysis giving rise to a linear chondroitin sulfate chain (Fig. 1). Some fucose units (approximately 25% of the total) remain after mild acid hydrolysis, but these are mostly non-sulfated residues, which occur as a cluster at the reducing end of the polysaccharide (34). The sulfated fucose branches resemble motifs present in sialyl-Lewis-containing glycans of leukocytes and tumor cells that are recognized by selectins. Therefore, we predicted that FucCS would inhibit selectin-mediated events, such as those observed in tumor metastasis and inflammation.

In the present study, we evaluated the ability of FucCS to block selectin-dependent interaction with sialyl-Lewis-containing glycans *in vitro* and investigate its effect on experimental models of metastasis and inflammation. The results show that FucCS is a potent inhibitor of P- and L-selectin-mediated interactions. The presence of the sulfated fucose branches is a fundamental requirement for the inhibitory activity of the invertebrate glycan. In addition, animal studies indicate that FucCS blocks tumor metastasis and reduces neutrophil recruitment to inflamed tissues, with only a minor effect on anticoagulation.

**EXPERIMENTAL PROCEDURES**

*Native and chemically modified glycosaminoglycans*. Porcine intestinal heparin (Mr= 12,000-15,000) was kindly provided by Patrick Shaklee (Scientific Protein Laboratories Inc., Milwaukee, Wisconsin, USA), or obtained from Sigma-Aldrich, Saint Louis, MO, USA. FucCS was extracted from the body wall of the sea cucumber *Ludwigothurea grisea* freshly collected from Guanabara Bay, Rio de Janeiro. The extraction and purification procedures were carried out as previously described (33-35). Removal of the fucose branches from the FucCS was performed by mild acid hydrolysis. In these experiments, the glycan (50 mg) was dissolved in 1.0 ml of 150 mM H2SO4, maintained at 100°C for
30 min, and the pH of the solution was adjusted to 7.0 with 0.3 ml of ice-cold 1.0 M NaOH.

Detailed structural analysis of the purified FucCS and of the product obtained after mild acid hydrolysis were performed using $^1$H-NMR spectra, determination of the monosaccharides composition and of the sulfate content, analysis of the disaccharides formed after digestion with chondroitin AC lyase and estimation of the molecular size by polyacrylamide gel electrophoresis, as previously described (34).

FucCS contains GlcUA, GalNH, Fuc and sulfate/total sugar on molar ratio of 1.0, 0.9, 1.2 and 0.7, respectively. Most of the fucose residues are removed after mild acid hydrolysis. The molar ratio of GlcUA, GalNH, Fuc and sulfate/total sugar changes to 1.0, 0.9, 0.3 and 0.6, respectively, in this derivative. The small amount of fucose units, which resists mild acid hydrolysis, are non-sulfated residues occurring as a cluster at the reducing terminal of the polysaccharide (34). Native FucCS is totally resistant to chondroitin AC lyase but after mild acid hydrolysis yields the following disaccharides after the action of the enzyme: ΔUA-GalNAc4/6diSO$_4$ (12%), ΔUA-GalNAc6SO$_4$ (53%), ΔUA-GalNAc4SO$_4$ (4%) and ΔUA-GalNAc (31%) (see also Ref. 34).

Comparison of the $^1$H and $^{13}$C-NMR spectra of native FucCS and of the product formed after mild acid hydrolysis indicates no significant desulfation of the central chondroitin sulfate core (34). The molecular sizes of the native FucCS and of the product formed by mild acid hydrolysis are 40 ad 8 kDa, respectively, as revealed by polyacrylamide gel electrophoresis (34).

**Mice.** C57BL/6 mice (6-8 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, MA, USA). For the LPS-induced lung inflammation experiments, C57BL/6 mice were obtained from the Instituto Nacional do Cancer, Rio de Janeiro, Brazil. Animals were maintained on a 12-hour light-dark cycle, and fed water and standard rodent chow ad libitum. All protocols were approved by the governmental administration of the institutions involved in the work.

**Binding assays.** Inhibition of selectin-sLe$^\alpha$ binding by the glycosaminoglycans was measured by coating sterile polystyrene 96-well ELISA plates (Corning Inc., Corning, NY, USA) at 4°C overnight with 200 ng of polyacrylamide-sLe$^\alpha$ (PAA-sLe$^\alpha$; Glycotech Corp., Rockville, MD, USA) in 100 μl of 50 mM sodium bicarbonate buffer, pH 9.5. Plates were blocked for 3 hours at 4°C with 200 μl/well of assay buffer containing 20 mM N-[2-hydroxyethyl] piperazine-N’-[2-ethanesulfonic acid], pH 7.45, 125 mM NaCl, 2 mM CaCl$_2$, 2 mM MgCl$_2$, and 1% protease-free BSA (Pentex; Miles Inc., Kankakee, IL, USA). Recombinant selectin-Ig chimeras were prepared as described previously (36) and were preincubated at 4°C for about 1 hour with peroxidase-conjugated goat anti-human IgG (1:1000 dilution in assay buffer, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). The final selectin-Ig concentrations were 2.7, 1.9, and 5.0 μg/ml for E-, L-, and P-selectin, respectively. The selectin-Ig/secondary antibody stock was aliquoted into tubes containing heparin, FucCS, buffer alone (positive control), 10 mM sodium EDTA (negative control), or anti-P-selectin or anti-E-selectin adhesion-blocking mAb (1 μg; Pharmingen, San Diego, CA, USA). The solutions (100 μl) were preincubated at 4°C for 30 minutes and added to ELISA plates. After 4 hours at 4°C, the plates were washed three times, followed by development with 2 μg/ml O-phenylenediamine dihydrochloride, 50 mM sodium citrate/sodium phosphate buffer, pH 5.2, and 0.03% H$_2$O$_2$. After 10 minutes, the peroxidase reaction was quenched by adding 50 μl of 4 M H$_2$SO$_4$. The absorbance at 490 nm was recorded using a microplate reader (Molecular Devices Inc., Menlo Park, CA, USA) equipped with SOFTmax software. Inclusion of a mAb to P-selectin, E-selectin of EDTA blocked binding by more than 90%. All the raw data were converted into percentages for comparative purposes using the formula: % of maximum = [(average of duplicates) − (negative control)]/[(positive control) − (negative control)] x 100.

**Glycosaminoglycan inhibition of LS180 binding to selectins.** The ability of heparin and FucCS to inhibit the adhesion of LS180 cells to immobilized P-, L- or E-selectins was examined as previously described, except that Calcein AM-loaded LS180 cells (ATCC CL187) were used (27). Results are expressed as percent of control binding, calculated using the following formula: 100 (glycan value – EDTA value) / (buffer alone value – EDTA value). Each glycan was tested in triplicate wells at each relative concentration.
In vivo inhibition of tumor cells-platelet interaction by FucCS. MC38 cells stably expressing GFP were prepared as described previously (16). Mice were intravenously injected with heparin (100 units) or FucCS (100 µg) followed by injection of 3 x 10⁵ of MC-38GFP cells 10 minutes later. Lungs were obtained for analysis 30 minutes after injection of tumor cells. Lung sectioning and staining were performed as described (37). To prevent collapse, lungs were injected via the trachea with OCT/PBS 1:1 solution and frozen in OCT compound (Tissue-Tek, Sakura, Torrance, CA). Frozen lung sections were stained with anti-CD41 antibody (Becton Dickinson, Mountain View, CA), followed by a staining with goat-anti-rat-Alexa 568 conjugated antibody (Invitrogen, Carlsbad, CA) and analyzed by immunofluorescence microscopy. The extent of platelet association with tumor cells was quantified by evaluating 20 view fields on 4 lung sections by 40x magnification.

Metastasis. Mice (6-8 weeks old) were intravenously injected with 3 x 10⁵ MC-38-GFP tumor cells via the tail vein. Some mice received intravenous injection of 50 µg of FucCS or PBS, 15 minutes prior to tumor cell injection. After 29 days, mice were anesthetized and perfused with PBS. Lungs were macroscopically evaluated for metastatic foci and further processed for quantification of metastasis by detection of GFP fluorescence as described previously (16). Briefly, lungs were homogenized in 2 ml of hypotonic buffer (20 mM Tris-Cl, pH 7.0) followed by addition of Triton X-100 to a final concentration of 0.5%. After 30 min on ice, the insoluble debris was sedimented (10,000 × g for 10 minutes), and 10 µl of supernatant diluted with Tris-Cl buffer (20 mM, pH 7.0) to a final volume of 100 µl was used for the fluorescence measurement with GENios ELISA reader (Tecan, Ex=485 nm and Em=535nm).

Thioglycollate-induced peritoneal inflammation. Mice were injected intraperitoneally with 2 ml of 3% thioglycollate broth (lot no. 54H4607; Sigma-Aldrich) or sterile pyrogen-free saline (Gibco BRL; Life Technologies Inc.). Five minutes later, the animals received intravenous injections of 0.2 ml sterile pyrogen-free saline with and without heparin or FucCS (0.2 or 0.5 mg/mouse). Mice were sacrificed after 3 hours, and the peritoneal cavities were lavaged with 8 ml of ice-cold PBS containing 3 mM EDTA to preventing clotting. Peritoneal cells were counted (Coulter Counter; Coulter Corp., Miami, FL, USA). The cells were also stained for 30 minutes at 4°C with FITC-conjugated rat anti-mouse Gr-1 mAb (Pharmingen) diluted in PBS containing 2.5% fetal bovine serum. After washing them three times with PBS, neutrophils were quantified on FACScan (Becton Dickinson Immunocytometry Systems, San Jose, California, USA) by gating cells expressing a high level of Gr-1 antigen (38).

LPS-induced lung inflammation. Male C57BL/6 mice weighing 20–25 g were injected intraperitoneally with 1.5 ml of sterile pyrogen-free saline with or without 0.25 mg of heparin, FucCS or dexamethazone, 1 hour before LPS inhalation. The inhalation procedure was previously described (39). After 3 hours, airspaces were washed with saline to provide 1.5 ml of bronchoalveolar lavage fluid (BALF). Total cells present in the BALF were counted with a Coulter counter ZM (Coulter Electronics, Hialeah, FL, USA). TNF-α levels were determined by a highly specific ELISA with a detection limit of 50 pg/ml.

Ex-vivo anticoagulant action measured by aPTT (activated Partial Thromboplastin Time). To determine the effect of FucCS on coagulation, the vena cava was isolated and cannulated with a micro catheter (Jelco, Johnson & Johnson Medical Inc., USA). Ten minutes after infusion of 100, 200 or 500 µg of FucCS, blood samples were collected by heart puncture into 3.8 % sodium citrate (9:1, v/v) for analysis of aPTT. At least 3 animals were used per group. The anticoagulant activity of FucCS was expressed as T<sub>i</sub>/T<sub>0</sub>, which is the ratio between the clotting time in the presence or absence of sulfated polysaccharide in the incubation.

RESULTS

FucCS blocks P- and L-selectin-mediated binding to sLe<sup>α</sup>. The interaction of FucCS with different selectins was evaluated by measuring the ability of the glycan to block binding of P- L- and E-selectin-Ig chimeras to immobilized PAA-sLe<sup>α</sup>.
Similar to heparin, FucCS inhibited the binding of P- and L-selectin-Ig chimeras to PAA-sLe\(^\alpha\), but had no effect on binding to E-selectin-Ig (Fig. 2). Inhibition occurred in a dose-dependent manner for P- and L-selectin-Ig, and at lower concentrations than that of mammalian heparin. Analysis of dose-response curves yielded IC\(_{50}\) values of 0.3 and 2.0 \(\mu\)g/ml for FucCS and heparin blocking P-selectin, respectively, whereas the corresponding values for blocking L-selectin were 0.25 and 0.5 \(\mu\)g/ml (Table 1). Removal of the sulfated fucose branches completely abolished the anti-P-selectin effect (Fig. 2), and also significantly reduced the anti-L-selectin effect.

To determine whether FucCS can block P- and L-selectin recognition of mucin-producing adenocarcinomas, we tested the inhibitory effect of FucCS on tumor cell adhesion to immobilized P- or L-selectin-Ig chimeric molecules. FucCS inhibited adhesion of LS180 adenocarcinoma cells to both mouse P- and L-selectins with an IC\(_{50}\) value of 10.4 \(\mu\)g/ml (Fig. 3 and Table 1). The inhibitory effect of heparin was much less potent yielding IC\(_{50}\) values of 40 \(\mu\)g/ml, respectively (Fig. 3 and Table 1). No inhibitory effect of FucCS or heparin was observed on the binding of LS180 cells to mouse E-selectin (data not shown). In summary, these data show that FucCS blocks both P- and L-selectin mediated interactions with sLe\(^\alpha\) and is more potent than heparin.

**FucCS reduces tumor cell-platelet association and prevents lung metastasis.** Platelet adhesion to tumor cells depends on activation of P-selectin expression on platelets, which permits binding to sLex containing mucins on the surface of tumor cells. In previous studies, we showed that heparin will block this interaction in vitro and in vivo (16). To investigate the potential of FucCS to inhibit platelet-tumor cell interactions in vivo, FucCS (100 \(\mu\)g) was intravenously injected 30 minutes before injection of MC-38 mouse adenocarcinoma cells. Mice were sacrificed 30 minutes later and sections of the lungs were analyzed for the presence of platelet-tumor cell aggregates (Fig. 4). At the doses used, FucCS and heparin (100 IU) significantly reduced tumor cell-platelet association, compared with saline-injected mice (Fig. 4).

These findings suggested that FucCS might also block tumor metastasis. To investigate this possibility, we injected 50 \(\mu\)g of FucCS into mice 15 minutes before injection of MC-38 cells. After 29 days, mice were sacrificed and the dissected lungs were analyzed for the presence of metastatic foci by measuring fluorescent GFP-tagged tumor cells and by counting tumor foci on the lung surface. FucCS significantly reduced lung metastasis in treated mice compared to the saline-treated animals by both methods (Fig. 5). Eight of nine animals showed a dramatic decrease in the number of metastatic foci, supporting the assumption that the anti-selectin effect of the FucCS correlates with its antimetastatic effect.

**FucCS prevents neutrophil recruitment in vivo.** Neutrophil recruitment occurs during acute inflammation and depends on both P- and L-selectins (40,41). To test if FucCS has anti-inflammatory activity, we examined thioglycollate-induced peritonitis, which is characterized by neutrophil infiltration into the peritoneal cavity. FucCS was injected intravenously at doses of 0.2 and 0.5 mg per mouse 5 minutes after thioglycollate injection. The half-lives of the heparin used (30) and FuCS (not published) are about the same (90-120 min). After 3 hours, when thioglycollate treatment induces about 120-fold neutrophil increase, the peritoneal cavity was washed with saline and neutrophils in the lavage fluid were counted. At the lower dose, FucCS inhibited neutrophil recruitment into the peritoneal cavity by \(
\text{about 60% compared with control, saline-treated animals (Fig. 6A). The same extent of inhibition was observed after intravenous injection of 0.5 mg of heparin. Increasing the dose by 2.5-fold produced further inhibition of neutrophil infiltration (p<0.005). Removal of sulfated fucose branches completely abolished the inhibitory effect of the polysaccharide (Fig. 6A).}

We also examined neutrophil recruitment into the alveolar compartment after inhalation of LPS, which induces a frank inflammatory response in the lungs. After 3 hours, airspaces were washed with saline and the number of neutrophils in the BALF was counted. Infiltration of neutrophils into alveolar compartment of LPS-treated mice was inhibited by 60 % by administration of 0.25 mg of FucCS compared with saline-treated animals (Fig. 6B). A similar inhibitory effect on neutrophil recruitment was observed when the animals were treated with the same dose of heparin. FucCS had no significant inhibitory effect on the production of TNF-\(\alpha\) in
the alveolar fluid (Fig. 7) indicating that the polysaccharide did not affect production of inflammatory mediators. In contrast, treatment with dexamethasone, a potent inhibitor of TNF-α production by macrophages, significantly reduced TNF-α to basal levels (p>0.001).

We also assessed the anticoagulant activity by aPTT assay of ex vivo plasma, 10 minutes after intravenous injection of FucCS (Fig. 8). Doses of 200 and 500 µg/mouse of FucCS produced a 1.4- and 2.5-fold increment in the plasma activated partial thromboplastin time (aPTT), respectively. Administration of 100 µg of FucCS only slightly increased aPTT, whereas heparin induced a ~9-fold increment.

DISCUSSION

Several studies have shown that the anti-selectin activity of heparin accounts for its high antimetastatic and anti-inflammatory effects (16-19,31,32,42). In the present work, we have shown that an unique sulfated polysaccharide isolated from the body wall of the sea cucumber *Ludwigithorea grisea* is a potent inhibitor of both P- and L-selectins. The polymer has a backbone like that of mammalian chondroitin sulfate (4-β-D-GlcA-1→3-β-D-GalNAc)ₙ, mostly 6-sulfated at the GalNAc unit, but substituted at the 3-O-position of the β-D-glucuronic acid residues with 2,4-disulfated α-L-fucopyranosyl branches (Fig. 1) (33-34). We provided evidence that the sulfated fucose branches are critical for the inhibitory activity of the FucCS on P- and L-selectins, whereas the sulfated galactosamine residues are not relevant. Previous studies indicated that the sulfated fucose branches are also required for the anticoagulant and antithrombotic activities of FucCS (34,35).

P-selectin glycoprotein ligand-1 (PSGL-1), a mucin present on the surface of leukocytes, binds P-selectin with high affinity (Kd ~ 300 nM) (43-48). The interaction is thought to involve two main regions in PSGL-1. One region consists of three sulfated tyrosine (TyrSO₃) residues along with a cluster of negatively charged amino acids at the N-terminal region of PSGL-1. A second region adjacent to the TyrSO₃ groups consists of an O-linked glycan containing sialic acid and fucose (sLe⁶). Together they yield a cooperative binding unit with enhanced avidity for P-selectin (49). Heparin, with its many sulfate groups including those at C6 of the glucosamine units, may selectively block the interaction of P-selectin with the TyrSO₃ cluster. Since FucCS also contains sulfate at C6 of the GalNAc units and the length of the central disaccharide unit is similar to the disaccharide unit in standard heparin (~10 Å) (50), it also could block the interaction of the TyrSO₃ cluster with P-selectin. The sulfated fucose side branches in FucCS could enhance its inhibitory activity by simultaneously interfering with the fucose/sialic acid oligosaccharide-mediated interaction.

Binding studies *in vitro* revealed that the dose of FucCS that inhibits 50% of P- and L-selectin binding to immobilized PAA-sLe⁶ and the attachment of LS180 cells to immobilized P- and L-selectins is much lower than that of heparin. These findings suggested that FucCS may have more efficient inhibitory effects on selectin-mediated events than heparin *in vivo*. In fact, the greater inhibition of platelet-tumor cell aggregation *in vivo* supports this hypothesis (Fig. 5). Similarly, a 10-fold lower dose of FucCS significantly reduced metastasis when compared to unfractionated heparin (UFH) (51). Interestingly, the anti-selectin effects of FucCS occur at doses much lower than that required for anticoagulation. Thus, the dose of FucCS to significantly elevate the plasma aPTT in mice was 500 µg, which is 5- and 10-fold higher than that required to inhibit platelet-tumor cell association and to abolish metastasis in mice, respectively (Fig. 8). Previous studies showed that high doses of FucCS, unlike heparin, abolished experimental thrombosis in rats without significant change in plasma aPTT (35). Thus, the therapeutic index for FucCS is much improved compared to heparin, which tends to inhibit metastasis and coagulation at comparable values (35,43).

The results obtained in the two experimental models of inflammation (thioglycollate-induced peritonitis and the LPS-induced lung inflammation) showed that FucCS inhibited neutrophil recruitment at the time-point studied. However, in the thioglycollate model, the FucCS was more potent than heparin, whereas in the LPS model both compounds showed comparable activity. The variation in dose response for heparin and FucCS in the two
systems could reflect differences in the mode of administration (intraperitoneal versus intravenous) and subsequent bioavailability or pharmacodynamics of the compounds. Although FucCS was able to inhibit neutrophil recruitment in these models, it did not decrease TNF-α production in the LPS model of inflammation, indicating that the anti-inflammatory effect of the invertebrate glycan involves mainly selectin-mediated events.

It has been reported (43) that the dose of UFH required for the anti-selectin effect is very close or higher than that needed for the anticoagulant action, which increases the hemorrhagic risk and makes the clinical use of UFH impractical as anti-metastatic and anti-inflammatory agent. Similarly, the use of low molecular weight heparin, which has a much lower hemorrhagic effect, is not a good alternative for UFH, since it is devoid of significant anti-selectin effects (43). Disaccharide primers composed by peracetylated \( \text{GlcNAC}\beta_{1,3}\text{Gal-naphthalene methanol} \) are in preclinical studies as a modified agent of \( \text{Sle}^\delta \) structures, that facilitate haematogenous metastasis (27), but as far as we know, no other heparin mimetics is currently being tested as an anti-metastatic agent. In this context, FucCS is a better alternative for UFH, since it has a higher activity, is devoid of significant bleeding effects and could be used without any further fractionation after purification. Another important aspect to take into account, when proposing the use of natural products from mammalian origin as therapeutic agents is the risk of contamination with pathogens. For example, the association of mammalian prion proteins with transmissible spongiform encephalopathy has recently restricted the use of bovine heparin. Since, heparin is obtained exclusively from porcine tissues, the risk of contamination with a prion protein or even a virus is still present. Based on its potency, better therapeutic index, less undesirable side effects, natural occurrence, high abundance, and ease of purification, intact FucCS could be used as a therapeutic agent in the treatment of cancer we recommend further testing of intact FucCS as potential substitute for heparin.
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FOOTNOTES

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1 The abbreviations used are: sLeX, sialyl Lewis x; sLea, sialyl Lewis a FucCS; fucosylated chondroitin sulfate; GlcA, glucuronic acid; GalNAc(6SO3), N-acetylgalactosamine bearing a sulfate group on carbon 6; PAA-sLea, polyacrylamide-sLea; GFP, green fluorescent protein; BALF, bronchoalveolar lavage fluid; TNF-alpha, tumor necrosis factor-alpha; aPTT, activated partial thromboplastin time; PSGL-1, P-selectin glycoprotein ligand-1. TyrSO3, sulfated tyrosine; UFH, unfractionated heparin.
Figure 1: Preponderant structure of the FucCS. The backbone of this glycan is made up by repeating disaccharide units of alternating β-D-glucuronic acid (A) and N-acetyl-β-D-glucosamine (B) the same structure as mammalian chondroitin-sulfate. However, in the case of the sea cucumber glycan, the β-D-glucuronic acid residues bear 2,4-disulfated fucose branches (C) at the 3-O-position. The branches are removed by mild acid hydrolysis (arrow). Most of the N-acetyl-β-D-galactosamine are 6-sulfated (53%), however small amounts of 4,6-disulfated (12%), 4-sulfated (4%) and non-sulfated (31%) are also found. Some fucose units (approximately 25% of the total) resist to mild acid hydrolysis, but these are mostly non-sulfated residues, which occur as a cluster at the reducing end of the polysaccharide (not shown in the Panel, see Ref. 34).

Figure 2: Inhibition of selectin-Ig binding to immobilized PAA-sLeα. Inhibition curves were obtained using FucCS (filled circles), heparin (filled squares) and de-fucosylated FucCS (deFucCS, open circles) (see methods). Each point represents the average of duplicate determinations, and the data are representative of three separate experiments. The points are the average value ± SEM.

Figure 3: Inhibition of LS180 cell binding to selectins. Adhesion of LS180 cells to immobilized selectin chimeras was measured in the presence of increasing concentrations of FucCS (filled circles) or heparin (open circles) (see methods). Each point represents the average of triplicate experiments, and the data are representative of three separate experiments.

Figure 4: In vivo inhibition of MC-38 cells-platelet interaction. Heparin (100 IU) or FucCS (100µg) was intravenously injected followed by an injection of MC-38GFP cells (see Methods). Platelet interactions with injected cells were evaluated in lungs from mice terminated 30 min after injection. Quantification of platelet-tumor cell interaction from 3 independent experiments. Horizontal bars represents medians, boxes represent percentiles 25 and 75, and vertical bars represents ranges. The observed differences were statistical significant as determined by one way ANOVA analysis (p< 0.01).

Figure 5: FucCS eliminates experimental metastasis. FucCS (50 µg) was intravenously injected followed by injection of 3 x 10⁵ MC-38-GFP tumor cells 15 minutes later (see Methods). Mice were terminated after 29 days. Dissected lungs were further macroscopically evaluated for the presence of metastatic foci (A) and processed for quantification of metastasis by detection of GFP fluorescence (B). Representative examples of dissected lungs from mice injected with FucCS or with PBS (C). Statistical significance was determined by Student’s t-test (p< 0.001).

Figure 6: Inhibition of neutrophil recruitment. (A) Thioglycollate-induced peritoneal inflammation. Heparin (0.5 mg/mouse), FucCS (0.2 or 0.5 mg/mouse) or de-fucosylated FucCS (deFucCS, 0.5 mg/mouse) were injected intravenously 5 minutes after thioglycollate was injected intraperitoneally. The number of Gr-1-positive granulocytes in the peritoneal cavity was quantified after 3 hours (see Methods). Significant difference in neutrophil counts: in the control mice that received PBS versus those injected with the indicated glycans (*); in mice injected with 0.5 mg of heparin or 0.2 mg of FucCS versus those injected with 0.5 mg of FucCS (***), and in mice injected with 0.5 mg of heparin or 0.2 mg of FucCS versus those injected with 0.5 mg of deFucCS (****) was determined. (B) LPS-induced lung inflammation. Heparin or FucCS (0.25 mg/mouse) were injected intraperitoneally 1 hour before LPS inhalation. After 3 hours, neutrophils were isolated and counted as described in Methods. *Significant difference in neutrophil counts in the control mice that received PBS versus those injected with the indicated glycans. Each bar represents the average value ± SD; N = 6. Statistical significance was determined by Student’s t-test (p< 0.001).
Figure 7: Inhibition of TNF-α production in LPS-induced lung inflammation. Heparin, FucCS or dexamethazone (Dexa) (0.25 mg) were injected intraperitoneally 1 hour before LPS inhalation. After 3 hours, TNF-α levels were determined by a highly specific ELISA assay. *Significant difference in the concentration of TNF-α in the bronchoalveolar fluid of control mice that received PBS versus those that inhaled LPS and/or were injected with FucCS. ** Significant difference in the concentration of TNF-α in the bronchoalveolar fluid of control mice that inhaled LPS and were injected with FucCS versus those that inhaled LPS and were injected with dexamethazone. Each bar represents the average value ± SD; N = 6. Statistical significance was determined by Student's t-test (p< 0.001).

Figure 8: Dose dependence for ex vivo anticoagulant action of FucCS. Ex vivo anticoagulant effects of different doses of FucCS (●-) or heparin (-○-) were determined by the activated partial thromboplastin time (aPTT) assays of ex vivo mouse plasma before (T₀) and 30 minutes (T₁) after intravascular administration of FucCS. Each point represents the average ± SD of triplicate experiments, and the data are representative of three separate experiments. T₀ = 22.1 ± 0.3 seconds. Conversion factor to convert T1/To into IU/mg = 0.53.
Table 1: Anticoagulant and inhibitory properties of heparin and FucCS

| Glycan  | aPTT (IU/mg) | Inhibition of Selectin-PAA-sLex Binding (IC50, µg/ml) | Inhibition of Tumor Cell-selectin Adhesion (IC50, µg/ml) |
|---------|--------------|------------------------------------------------------|--------------------------------------------------------|
|         |              | P-selectin | L-selectin                           | P-selectin | L-selectin |
| Heparin | 193          | 2.0        | 0.5                                  | 40         | 40         |
| FucCS   | 40<sup>a</sup> | 0.3        | 0.25                                 | 10.4       | 10.4       |

<sup>a</sup> Value obtained from reference 34.
FIGURE 1

\( R1 = 65\% \text{ SO}_3^-; 35\% \text{ H} \)

\( R2 = 16\% \text{ SO}_3^-; 84\% \text{ H} \)
FIGURE 2

[Graph showing inhibition of selectins by different inhibitors]
FIGURE 3
FIGURE 4
FIGURE 5
FIGURE 6
FIGURE 7
FIGURE 8