

Heparin Octasaccharides Inhibit Angiogenesis *In vivo*

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Abstract **Background:** In previous experiments, we showed that heparin oligosaccharides inhibit the angiogenic cytokine fibroblast growth factor-2. Here, we present the first *in vivo* study of size-fractionated heparin oligosaccharides in four models of angiogenesis that are progressively less dependent on fibroblast growth factor-2.

Experimental Design: Heparin oligosaccharides were prepared using size-exclusion gel filtration chromatography and characterized through depolymerization and strong anion exchange high-performance liquid chromatography. Size-defined oligosaccharides (20 mg/kg/d) were given to mice bearing s.c. sponges that were injected with fibroblast growth factor-2 (100 ng/d). After 14 days, octasaccharides and decasaccharides reduced the microvessel density to levels below control. In a second experiment, HEC-FGF2 human endometrial cancer cells that overexpress fibroblast growth factor-2 were implanted in a hollow fiber placed s.c. *in vivo*. Oligosaccharides were given at 20 mg/kg/d for 2 weeks and the data again showed that octasaccharides significantly reduced microvessel density around the fiber ($P = 0.03$). In a more complex model, where angiogenesis was induced by a broad spectrum of growth factors, including vascular endothelial growth factor, we implanted H460 lung carcinoma cells in hollow fibers and treated the animals with oligosaccharides at 20 mg/kg/d over 3 weeks. Octasaccharides reduced the microvessel density to that of control. Preliminary investigation of 6-*O*-desulfated heparins showed that these also had antiangiogenic activity.

Results: Finally, we examined the inhibitory potential of hexasaccharides and octasaccharides given at 20 mg/kg/d and these inhibited the growth of H460 lung carcinoma *in vivo*. At clinically attainable concentrations, significant anticoagulation (activated partial thromboplastin time, anti-factor Xa, and anti-factor IIa) was not observed *in vitro* unless species containing ≥ 16 saccharide residues were investigated.

Conclusions: Thus, our preclinical data show that heparin octasaccharides represent novel antiangiogenic compounds that can be given without the anticoagulant effects of low molecular weight heparin.

Angiogenesis was validated recently as a target for cancer treatment in a randomized trial, which showed that patients with metastatic colorectal cancer survived longer if their chemotherapy was given with an antibody to vascular endothelial

growth factor (VEGF; ref. 1). The process of angiogenesis is complex in that it is dependent on the balance of angiogenic and antiangiogenic factors; therefore, targeting other angiogenic cytokines in addition to VEGF might improve the efficacy of antiangiogenic and thus anticancer treatments.

Many angiogenic proteins are dependent on heparan sulfate for their biological activity, highlighting a potential strategy for pharmacologic inhibition. Heparan sulfate is a linear polysaccharide that consists of alternate residues of *N*-substituted glucosamine and a hexuronic acid (iduronic or glucuronic acid). The molecule has been implicated in several cancer-associated phenotypes and processes (2). Heparan sulfate is found on the surface and in the extracellular matrix of nearly every tissue-forming cell in the body where it plays a critical role in growth factor regulation, mediated principally by the interactions between sulfated domains in the heparan sulfate chain and protein ligands (2). Indeed, specific enzymatic modulation of heparan sulfate *in vivo* can radically alter tumor growth and metastasis, confirming the relevance of this family of molecules to oncology (3).

The prototypic heparan sulfate-dependent growth factor is fibroblast growth factor (FGF)-2, although several isomers of

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VEGF bind and are dependent on heparan sulfate (4, 5). Particular sequences of heparan sulfate have been identified, which bind the cytokine (6), whereas others bind its receptor (7, 8). As FGF-2 is angiogenic (9, 10), antagonists of heparan sulfate function should be antiangiogenic. In previous studies, we (11) and others (12, 13) showed that heparin oligosaccharides inhibited FGF-2-induced mitogenesis and motogenesis *in vitro*. Although earlier data focused on very short saccharide species containing up to six saccharide residues (14), here we present the results from the first study to investigate the effects of a broad range of size-fractionated heparin oligosaccharides *in vivo*.

Several attempts to generate saccharide inhibitors of this mechanism have yielded molecules that have been associated with dose-limiting anticoagulation (15), thrombocytopenia (16), or other toxicities. Thus, in this study, we also present *in vitro* data on the safety of short oligosaccharides with respect to anticoagulation at clinically relevant concentrations.

Materials and Methods

Preparation and compositional analysis of heparin oligosaccharides. Heparin oligosaccharides were obtained from tinzaparin sodium (Innohep, Leo Pharmaceuticals, Princes Risborough, United Kingdom) by size-exclusion chromatography using a Bio-Gel P10 gel filtration column (240 × 2 cm) run at 9 mL/h in 0.25 mol/L NH₄HCO₃. Three-milliliter fractions were collected. Eluted material was monitored at 232 nm and peaks of size-defined oligosaccharides were pooled, exhaustively freeze-dried, dissolved at a concentration of 100 µg/µL in 1.3% potassium acetate in ice-cold ethanol, and incubated at 4°C for 1 hour. The precipitated oligosaccharides were recovered by centrifugation at 10,000 rpm for 5 minutes and the pellets were dried by centrifugal evaporation. The pellets were weighed and stored at -20°C until required.

Disaccharide analysis by strong anion exchange high-performance liquid chromatography. Oligosaccharide pellets were dissolved in physiologic saline at a concentration of 100 µg/µL, and 1-µL suspension was taken for disaccharide analysis. The heparan sulfate oligosaccharides were completely depolymerized to disaccharides with a mixture of 2 milliunits heparinase I to III in heparinase buffer [100 µL; 0.1 mol/L sodium acetate, 0.1 mol/L calcium acetate (pH 7)] incubated overnight at 37°C.

The resulting disaccharides were then resolved by high-performance liquid chromatography on a ProPac PA1 analytic column (4 × 250 mm) using a linear gradient of 0 to 1 mol/L NaCl at 1 mL/min over 45 minutes in Milli-Q water (pH 3.5). The elution positions of specific disaccharides detected by absorbance at 232 nm were established by comparison with known standards.

In vivo experiments. All animal experiments were conducted according to UK Home Office guidelines on animal experiments, with all protocols approved by the UK Home Office.

All *in vivo* assays were conducted in a double-blind fashion so that neither the scientist who injected the saccharides nor the person responsible for counting microvessel density (MVD) knew the identity of the oligosaccharide species until all data had been collected.

The sponge implant assay. The assay was done as described previously (17). Circular polyether (Caligen Foam Ltd., Accrington, United Kingdom) sponge discs measuring 8 × 4 mm were cut using a cork borer. The discs were sterilized by boiling in distilled water for 10 minutes and overnight UV irradiation in a laminar flow hood.

Eight cohorts of five female C57BL/6J mice were anesthetized and a sponge was implanted s.c. through a 1-cm midline incision. Subsequently, the animals were housed singly with free access to food and water. The general condition of the animals was monitored daily.

The day of sponge insertion was taken as day 0. Commencing day 1, animals were treated daily with 20 mg/kg oligosaccharide (in 1% bovine serum albumin) plus 100 ng FGF-2 (in 1% bovine serum

albumin; experimental arms), FGF-2 only, or saline only (control arms) injected s.c. through the dorsal skin into the sponge. Size-defined heparin oligosaccharides ranging in size from hexasaccharides to saccharides containing 14 residues were given for 7 days in the first experiment and 14 days for the second.

On the day following the last injection (day 15), the animals were sacrificed and the sponges were removed. The dissected sponges were fixed overnight in 10% neutral formalin and processed for paraffin embedding and quantitation of MVD by immunohistochemical detection of von Willebrand factor.

The hollow fiber assay. Polyvinylidene difluoride fibers (Spectra/Por, Spectrum Laboratories, Huntingdon, Cambridge, United Kingdom), with an internal diameter of 1 mm and a molecular weight cutoff of 500 kDa, were cut to length, rehydrated, and sterilized by washing in 70% ethanol for 72 hours and flushing through and immersing in distilled water followed by autoclaving at 121°C for 20 minutes. Fibers were then flushed and immersed in complete RPMI 1640 (Sigma, Hertfordshire, United Kingdom).

H460 lung carcinoma cells that have high VEGF expression when loaded in hollow fibers *in vitro* (data not shown) were obtained from the American Tissue Type Collection (LGC Promochem, Middlesex, United Kingdom), whereas FGF-2-overexpressing human endometrial cancer HEC-FGF2 cells were produced by transfection (9). Cells were maintained as monolayer cultures in RPMI 1640 supplemented with fetal bovine serum (10%), sodium pyruvate (2 mmol/L), and L-glutamine (2 mmol/L; all from Sigma) at 37°C in a humidified 5% CO₂ environment. Cells were harvested by trypsinization and resuspended in RPMI 1640 at 2.5 × 10⁵/mL. This cell preparation was then used to load the fiber. Hollow fibers were then cut to a uniform length of 1.5 cm, which were heat sealed at both ends. The fibers were then transferred to six-well plates containing sterile medium and incubated at 37°C for 2 hours to allow cells to equilibrate within the fibers before transplantation.

Male NCR-Nu strain nude mice (National Cancer Institute, Bethesda, MD) ages 6 to 8 weeks were used. Mice were randomly allocated into experimental groups of five animals. Under a brief inhalational anesthesia, individual fibers were implanted s.c. on the left or right dorsal flanks using a trocar. Following recovery, the mice were housed together with easy access to food and water.

The day of fiber implantation was taken as day 0. Beginning on day 1, the designated treatment (oligosaccharide/saline) was given i.p. on a daily basis. The animals received daily injections of oligosaccharide (6-14 residues) at 20 mg/kg/d for up to 23 days and the mice were monitored daily for their general condition. On the day following the end of treatment, the mice were sacrificed and the fibers were removed *in situ* with a surrounding margin of s.c. tissue. The fibers were fixed overnight in 10% formalin and processed for paraffin embedding and evaluation of angiogenesis by immunohistochemistry. Some fibers were retained to assess cell survival using a modified version of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

Subcutaneous xenograft model. H460 lung carcinoma cells (10⁶) were implanted s.c. in nude mice ($n = 5$ per variable). Hexasaccharides or octasaccharides at 20 mg/kg/d or saline were injected s.c. each day for up to 3 weeks commencing 2 days before implantation and the tumors were measured daily. Animals were sacrificed if toxicity was observed or if the tumors exceeded the permitted tumor volume.

In vitro analysis of cytotoxicity. To assess whether the oligosaccharides affected angiogenesis or if the observed effect was secondary to a cytotoxic effect on cells contained in the hollow fibers, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used (18). Hollow fibers containing 1 × 10⁶ H460 cells/mL were placed in wells containing 2 mL complete medium, which was replaced 24 hours before assay. To each well were added 1 mL sterile 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma; 1.25 mg/mL) and 2 mL complete medium. Plates were then incubated for 4 hours at 37°C. Subsequently, 2.5% protamine sulfate (2 mL) was added and the mixture was stored at 4°C in the dark for at least 2 to 4 hours. This was

then replaced with fresh protamine sulfate and the fibers were then placed in a dry well in a laminar flow hood to dry out the fibers. Subsequently, DMSO (300 μ L) was added to each well and the plate was shaken gently for 4 hours at room temperature to dissolve the formazan product. The solution (190 μ L) was then transferred to a 96-well plate and the absorbance at 540 nm was read using the automatic plate reader.

Immunohistochemical assessment of the microvasculature. Sections of paraffin-embedded tissue were cut and hydrated using a graded alcohol series. The sections were then stained using rabbit anti-von Willebrand factor A0082, Strept-ABC complex/AP K0391, rabbit immunoglobulin X0903 (all from DAKO, Ely, Cambridge, United Kingdom), and alkaline phosphatase substrate kit SK-5100 (Vector Laboratories, Peterborough, United Kingdom).

In the sponge assay, microvessels were counted in all fields across the entire sponge area at $\times 400$ magnification (field size, 0.15 mm²). Three nonserial sections per sponge were used. This was done independently by two observers who were unaware of the treatment assignments. With the hollow fiber assay, microvessel counts were done as described above by one observer in two nonserial sections. Section-to-section variance within the same animal was small and multiple section analysis did not add to the precision of comparisons. The concordance between counts of two observers was predictable in the sponge assay; therefore, a single observer was used for counting in the hollow fiber assay.

Anticoagulation. Size-defined oligosaccharides were added to platelet-free normal human plasma at concentrations up to 40 μ g/mL. Activated partial thromboplastin time was determined using the standard clinical assays available in the hematologic anticoagulation laboratory. Anti-factor Xa activity was determined by an in-house clotting assay. Anti-factor IIa activity was measured by a chromogenic method (Spectrolyse Heparin Anti-Factor IIa, BioPool, Bray, Ireland). Activities were compared with unfractionated tinzaparin.

Statistical methods. The primary analytic tool was a linear mixed-effects model. Such models are appropriate when we have more than one random term in an experiment compared with a classic setup with just a single error term. The data were analyzed on a logarithmic scale. The significance of terms in each model was assessed with likelihood ratio tests. If there were significant differences between treatments in an overall comparison, then pairwise comparisons for each treatment group were made against the positive control and back transformed to be expressed on a ratio scale.

The sponge assay was the most complicated model. In this model, there were three fixed effects (f ; treatment, observer, and treatment \times observer interaction) and two random effects (r ; sponge pooling and experimental error). The overall test for treatment effect was therefore calculated using the following variables: Response: $\log_{10} V_c$ (vessel count) = treatment effect (f) + observe effect (f) + treatment \times observer interaction (f) + pooling effect (r) + experimental error (r).

To simplify the statistical model, the treatment \times observer interaction was discounted. Although interobserver variability in vessel counts between multiple observers was expected, it was unlikely to have a marked effect on the overall result.

In the hollow fiber assay with H460 cell lines, again there were two fixed and two random effects. Response: $\log_{10} V_c$ = treatment effect (f) + occasion effect (i.e., HF1 versus HF2; f) + animal-to-animal variation (r) + experimental error (r).

Results

Oligosaccharide preparation and characterization

Oligosaccharides were prepared from commercially available low molecular weight heparin by size-exclusion chromatography as described above. Each preparation of size-defined oligosaccharides was then completely degraded to component disaccharides, which were identified through strong anion exchange high-performance liquid chromatography. The results are summarized in Table 1. The majority of disaccharides

Table 1. Characterization of oligosaccharide composition

	No. oligosaccharide residues						
	4	6	8	10	12	14	16
% Disaccharide composition of oligosaccharides							
UA-GlcNAc	1.9	3.1	3.0	1.5	4.1	7.3	7.3
UA-GlcNS	0.0	0.0	0.9	1.3	1.8	3.2	4.0
UA-GlcNAc(6S)	0.0	0.0	1.6	1.2	1.5	3.5	3.9
UA-GlcNS(6S)	7.7	11.0	8.7	9.2	8.8	8.5	9.7
UA(2S)-GlcNS	8.9	7.5	8.3	8.3	7.1	7.9	9.5
UA(2S)-GlcNS(6S)	81.5	78.0	77.0	78.4	76.6	69.6	65.5
% Disaccharides sulfated at designated site							
<i>N</i> -sulfation	98.1	96.5	94.9	97.2	94.3	92.7	88.7
2- <i>O</i> -sulfation	90.4	85.5	85.5	86.7	83.7	77.5	75.0
6- <i>O</i> -sulfation	89.2	89.0	87.3	88.8	86.9	81.6	79.1

NOTE: Oligosaccharides were prepared by size-exclusion gel filtration chromatography and depolymerized with heparinase I to III and the component disaccharides were identified through strong anion exchange high-performance liquid chromatography. Rows represent the identity of component disaccharides and columns represent the length of saccharide that were analyzed. UA, uronic acid; GlcNAc, *N*-acetylated glucosamine; GlcNS, *N*-sulfated glucosamine; 6S, 6-*O*-sulfate; 2S, 2-*O*-sulfate. The percentage of a particular sulfation was calculated by adding the percentages of relevant component disaccharides.

present in the oligosaccharides were trisulfated, suggesting that overall these compounds were highly sulfated species. The analysis shows that the composition of saccharides was relatively homogeneous particularly when molecules of between 4 and 12 saccharide residues were considered.

Sponge model of fibroblast growth factor-2 angiogenesis

Histologic findings. The sponge model allows the investigation of angiogenesis driven by a defined molecule, which can be injected into the sponge. We tested the potential of oligosaccharides to inhibit FGF-2-induced angiogenesis in this model. Sponges were implanted s.c. and then injected on a daily basis with either saline or FGF-2 (100 ng/d). FGF-2 was either given alone or together with oligosaccharides (20 mg/kg/d).

After removal, examination revealed that the sponges were enveloped in granulation tissue and often accompanied by some skeletal muscle and s.c. adipose tissue. Histologically, sponges could be readily distinguished as clear triangular structures, whereas microvessels were principally found in the surrounding granulation tissue.

Inflammatory infiltrates were also seen within the substance of the sponge as were RBC from ruptured vessels. Granulation tissue encroached into the sponge and microvessels were identified within the center of the sponge. Microvessels in the sponge substance and surrounding granulation tissue were by definition new vessels as these were not present before sponge implantation; they therefore represented neoangiogenesis and were the vessels that were counted.

Immunolabeling for von Willebrand factor revealed that the new vessels were disorganized with thin walls, a discontinuous basement membrane, and a sparse covering of pericytes and smooth muscle. Individual endothelial cells were clearly identified and occasional RBC could be seen within the lumen.

Figure 1A to D shows low-power and high-power photomicrographs at low and high MVD taken from different sponges.

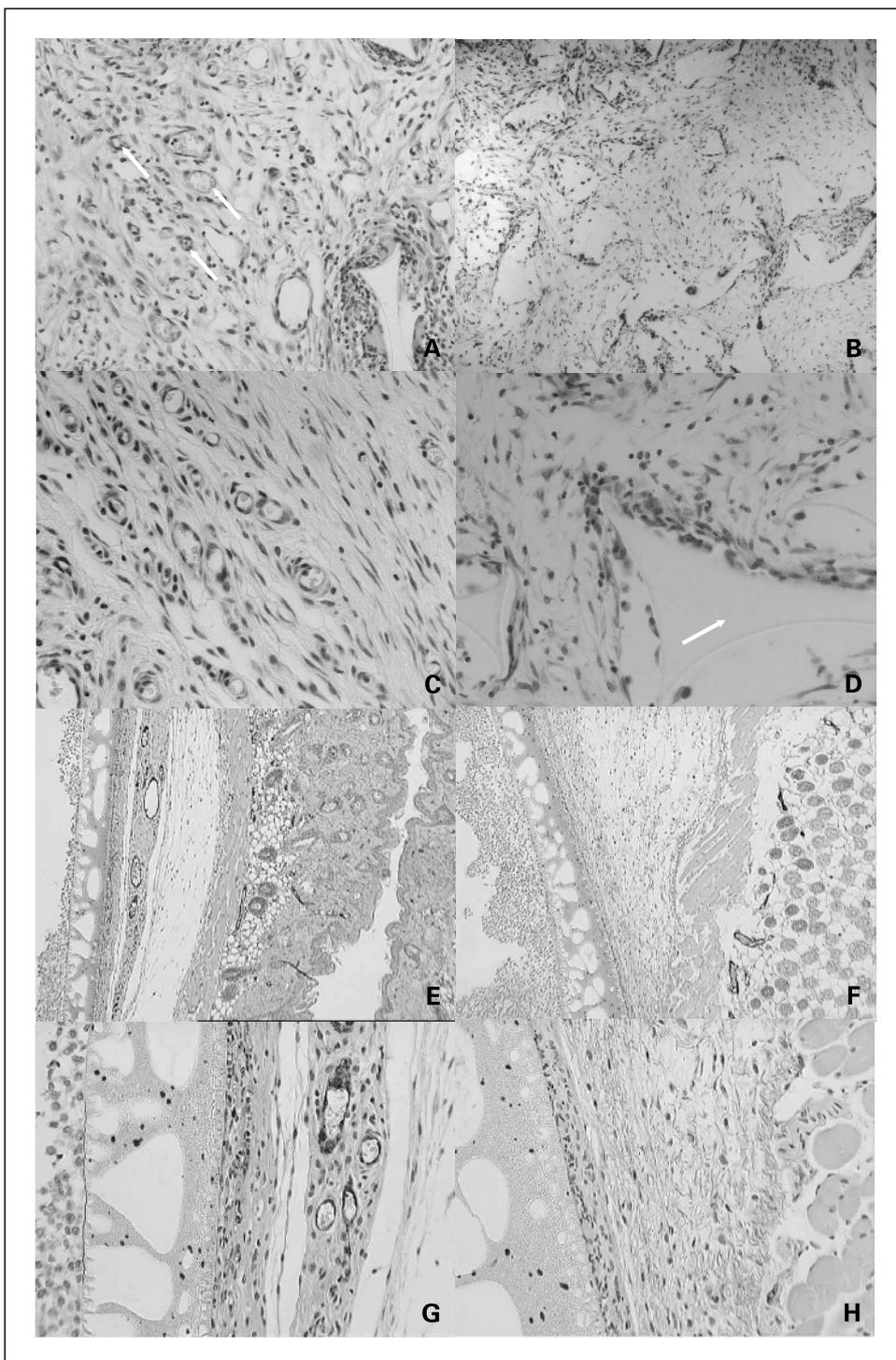
Microvessel quantitation. The total microvessels in three nonserial sections at 400-fold magnification were counted. By comparing microvessel counts between sponges treated with heparin-derived oligosaccharides and FGF-2 alone, the degree of inhibition of neoangiogenesis was quantified. Figure 2A shows the data from two experiments in which octasaccharides and decasaccharides reduced, by >95% confidence interval, the MVD in comparison with the positive control.

Hollow fiber model of angiogenesis

The sponge model of angiogenesis allows the investigator to direct and therefore investigate angiogenesis with reference to a single molecule. However, human tumor angiogenesis is dependent on several growth factors and we therefore tested the inhibitory potential of the oligosaccharides on cell-induced angiogenesis where the cells were contained in hollow fibers so that the processes of invasion and angiogenesis can be separated (19).

Although the hollow fiber model has been used to investigate anticancer drugs under the National Cancer Institute drug

Fig. 1. Representative histologic sections from sponge and hollow fiber assays. Left, high MVD tissues; right, low MVD tissues. *A* to *D*, sponge model. *A* and *B*, low-power fields; *C* and *D*, high-power fields. Yellow arrows, vessels; green arrow, sponge. *E* to *H*, hollow fiber model of angiogenesis. *E* and *F*, low-power fields; *G* and *H*, high-power fields.



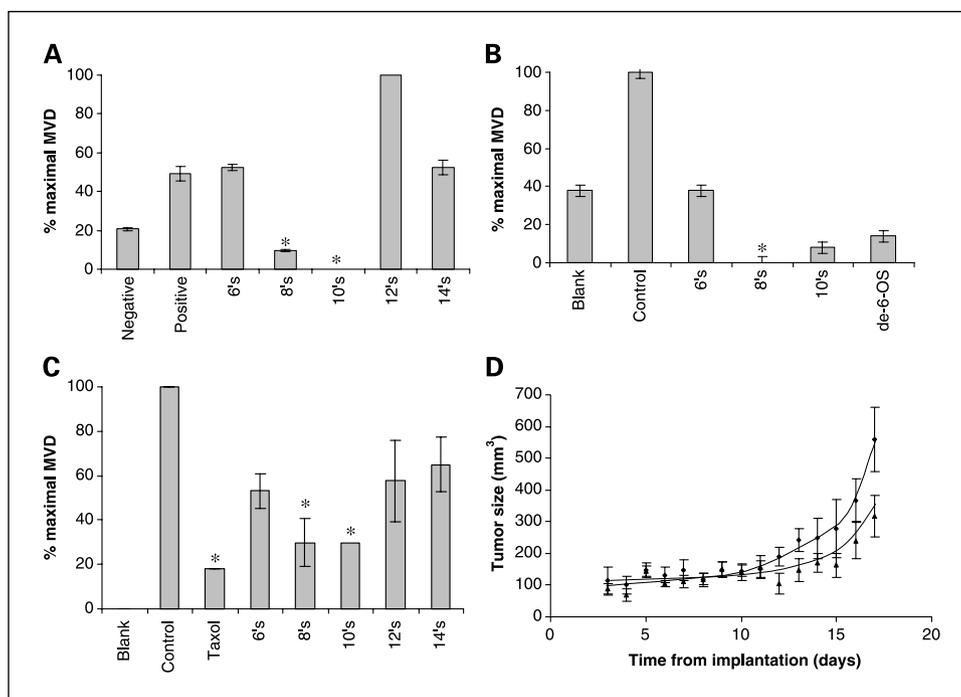


Fig. 2. Inhibition of angiogenesis by size-defined oligosaccharides. *A*, oligosaccharide-mediated inhibition of FGF-2-induced angiogenesis in the sponge model *in vivo*. Sponges were implanted s.c. in mice and injected with FGF-2 (100 ng/d) with size-defined oligosaccharides (20 mg/kg/d; usually ~0.4 mg). At days 8 and 15, the sponges were excised, formalin fixed, and processed for paraffin embedding and immunohistochemistry. The MVD was then counted. Representative of two experiments. Y-axis, percentage of positive control MVD. The negative control represented the MVD seen in sponges injected with saline, whereas the positive control were sponges injected with FGF-2 alone. All experiments were done twice with cohorts of five mice per experimental condition and were conducted in a double-blind fashion. Columns, mean; bars, SE. *, data points that exceeded the 95% confidence intervals of the assay. *B* and *C*, oligosaccharide-mediated inhibition of HEC-FGF2-induced (*B*) and H460-induced (*C*) angiogenesis in hollow fibers. Hollow fibers containing human endometrial cancer cells transduced to overexpress FGF-2 (*B*) or H460 human lung carcinoma cells (*C*) were implanted s.c. in nude mice, which were then treated with size-defined oligosaccharides or controls for 2 weeks. The tissues were then excised and the MVD was counted in high-power fields. The blank control represents an empty hollow fiber, whereas the positive control samples contained H460, but the mice were treated with saline. As a further control, some animals were treated with paclitaxel (20 mg/kg/d i.p. between days 7 and 11), a microtubule inhibitor that has antiangiogenic capacity. The experiment was terminated on day 24. In addition, the viability of H460 cells was assessed after the experiment and was shown to be preserved (data not shown). Each experiment was done twice in a double-blind manner and each specimen was counted. Columns, mean of two experiments; bars, SE. Y-axis, percentage of vessel density in the positive control. *, data points that exceeded the 95% confidence intervals of the assay. *D*, inhibition of H460 lung carcinoma growth *in vivo*. H460 lung carcinoma cells were implanted s.c. in nude mice ($n = 5$ per arm) and allowed to grow. At the time of tumor implantation, the animals were treated with saline (◆), hexasaccharides (data not shown as they were not significantly different from control), or octasaccharides (▲). Y-axis, percentage change in tumor volume from the start of the experiment in animals up to day 17. Points, mean; bars, SE.

evaluation program, adaptations to the protocol have been made to facilitate the study of angiogenesis (20). Modifications include reducing the number of fibers transplanted at one site and reducing the fiber length, both to reduce inflammation.

Before embarking on the angiogenesis assays, we investigated whether the oligosaccharides were cytotoxic to exclude a secondary effect on angiogenesis mediated through cell death. Using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay to measure cell viability, these studies showed that, when hollow fibers were incubated *in vitro* in concentrations of up to 400 $\mu\text{g}/\text{mL}$, none of the oligosaccharides were cytotoxic, whereas under the same conditions doxorubicin was cytotoxic at 52 $\mu\text{g}/\text{mL}$ (data not shown).

The duration of the assay is based on preliminary studies where the MVD surrounding hollow fibers loaded with H460 cells was analyzed at different time points (20). This showed that there was a statistically significant increase in angiogenesis by week 2, which was maintained at week 3, whereas at week 1 there was no difference between empty and cell-containing hollow fibers (data not shown).

Histology. When excised, the hollow fibers were encompassed in organizing granulation tissue and a rim of surrounding s.c. tissue (Fig. 1E-H). On gross inspection of the fibers

in situ, no obvious differences in surrounding vasculature were observed, although more pronounced vascularity was observed at the end of the fibers than along the shaft. Following fixation and paraffin embedding, the fibers were cut along the longitudinal axis.

As before, the average MVD was measured by immunohistochemical staining for von Willebrand factor. The hollow fibers were enveloped in well-organized granulation tissue that was particularly florid at the ends of the fibers. This correlated with the MVD, with a greater proportion of microvessels being found at the ends of the fibers than along the shaft.

The morphologic characteristics of the microvessels were similar to those seen in the sponge assay. Branching of new vessels was also noted. Components of host adipose and skeletal muscle tissue were also present at the periphery of the sections.

Microvessel quantitation. Vessels in the granulation tissue closely applied to the hollow fibers were considered for counting as these were by definition new vessels. Microvessels within the host adipose and skeletal muscle tissue were not counted as these were likely to represent preexisting vasculature. Two nonserial sections per fiber were counted.

In initial experiments, we examined the effect of oligosaccharides in a system partially dominated by FGF-2. Here,

hollow fibers containing human endometrial cancer cells, which had been transduced to overexpress FGF-2 (9), were placed s.c. in mice treated with s.c. daily doses of oligosaccharides. The results, which reflect the more relevant cell-based induction of angiogenesis, are shown in Fig. 2B and confirm again that heparin octasaccharides and decasaccharides significantly inhibited angiogenesis in comparison with the positive control fiber ($P < 0.05$). In this experiment, we also studied the potential of de-6-*O*-sulfated heparin species to inhibit FGF-2, the rationale being that at least one 6-*O*-sulfate group is needed for a heparan sulfate-FGF-2 complex to bind and activate the signal transducing receptor (7, 8). Based on prior *in vitro* studies of heparan sulfate structure, a de-6-*O*-sulfated heparin sequence should retain the ability to bind FGF-2 but should be unable to support the formation of a trimolecular signaling complex between FGF-2, heparan sulfate, and the signal transducing receptor. The data support this hypothesis in that this preparation also inhibited angiogenesis. Although variation across the assay precluded cross-species comparisons, the 95% confidence intervals were exceeded when the effect of the octasaccharides was compared with positive control.

To investigate the inhibitory potential of the oligosaccharides in a more complex situation, we investigated their effect on angiogenesis when H460 human lung carcinoma cells contained in a hollow fiber induced the process. In this case, angiogenesis was no longer driven predominantly by FGF-2; rather, the cytokine milieu produced by the H460 cells, including VEGF, was responsible for the process. The data are shown in Fig. 2C. Here, we compared the efficacy of the oligosaccharides against a known antiangiogenic regimen (paclitaxel). Whereas paclitaxel inhibited angiogenesis by 80%, the octasaccharides and decasaccharides inhibited angiogenesis by 69%, a favorable comparison considering the low toxicity of oligosaccharides. The other oligosaccharide species were less potent inhibitors.

H460 lung carcinoma *in vivo*

The above data showed that heparin oligosaccharides inhibited FGF-2-induced angiogenesis in both the sponge and the hollow fiber models. Oligosaccharides also inhibited angiogenesis induced by H460 lung carcinoma cells. We therefore investigated the effect that the oligosaccharides had on H460 lung carcinoma tumor growth *in vivo*. Figure 2D shows that octasaccharides when given at 20 mg/kg/d were associated with a 3-day retardation of growth ($P = 0.2$).

Anticoagulation

One of the principal problems with dose escalation of saccharide-based compounds in phase I trials is overanticoagulation. We therefore investigated the ability of size-defined oligosaccharides to inhibit relevant parts of the coagulation cascade in normal human plasma using clinically validated assays. These included factor IIa, factor Xa, and activated partial thromboplastin time (Fig. 3A), the rationale being that heparin enhances the activity of antithrombin by inducing a transformation in the antithrombin molecule (21), allowing faster inhibition of factor Xa (Fig. 3B) and factor IIa (Fig. 3C).

Our data suggest that overanticoagulation is unlikely to occur when species less than 14 residues were employed. At concentrations of 20 $\mu\text{g}/\text{mL}$, oligosaccharides with a chain length of up to 12 showed little prolongation in activated

partial thromboplastin time. The effect of oligosaccharides composed of 16 residues was greater but still well within the therapeutic range for unfractionated heparin. In the anti-factor Xa test, very short oligosaccharides (1–3, 9, 19) showed little activity even at 40 $\mu\text{g}/\text{mL}$. The longest chain tested (16 saccharides) showed significant overanticoagulation at 30 $\mu\text{g}/\text{mL}$. Those with chain lengths from 8 to 14 showed anti-factor Xa activity within the therapeutic range for low molecular weight heparin even at the 40 $\mu\text{g}/\text{mL}$ concentration. The effect of oligosaccharides up to chain length 14 on the assay of anti-factor IIa activity was minimal at concentrations up to 40 $\mu\text{g}/\text{mL}$. However, at a concentration of 40 $\mu\text{g}/\text{mL}$, the longest chain studied was associated with anticoagulation that was greater than the top of the therapeutic range for unfractionated heparin. These data suggest that potentially toxic effects on the coagulation cascade would only be seen

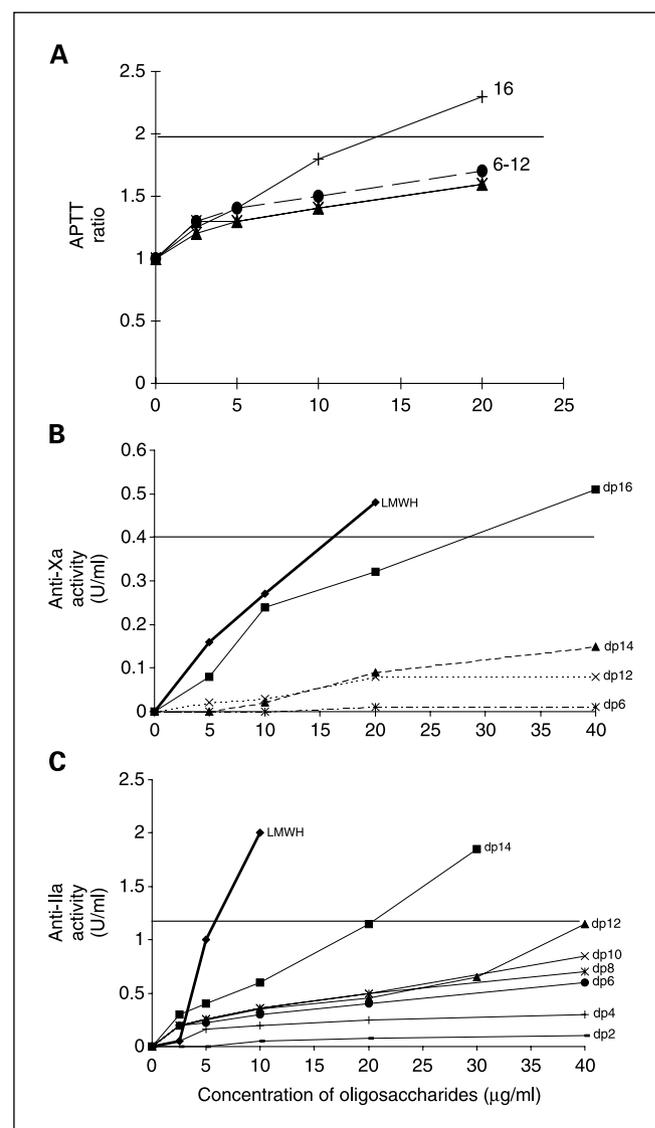


Fig. 3. Oligosaccharide-induced anticoagulation. Oligosaccharides of defined lengths and low molecular weight heparin (LMWH) at indicated concentrations (X axis) were added to human plasma and their effects on activated partial thromboplastin time (APTT; A), anti-factor Xa (B), and anti-factor IIa (C) were determined by standard laboratory assays. Duplicate assays at each concentration were within 5%. Horizontal lines, upper limit of clinically acceptable anticoagulation.

once high doses of species shorter than 14 residues were studied. Thus, it should be possible to escalate the doses of these species to levels in excess of those currently given to patients, as longer anticoagulating species would not be given.

Discussion

Several previous studies showed that heparin oligosaccharides inhibited the biological activity of FGF-2 *in vitro* (11, 13), yet very few studies have tested their potential *in vivo* (14) and none have explored the size-inhibition relationship for larger heparin oligosaccharides *in vivo*. Here, we have used several different experimental models to show that heparin oligosaccharides have antitumor activity and antiangiogenic activity mediated, at least in part, through the inhibition of FGF-2.

We investigated a series of models in which angiogenesis was sequentially less dependent on FGF-2. We showed that, whereas octasaccharides completely abrogated angiogenesis in the models most dependent on FGF-2 [the sponge model (Fig. 2A) and the hollow fiber containing FGF-2-expressing cells (Fig. 2B)], less profound effects were seen in the more complex models [hollow fiber containing H460 cells (Fig. 2C) and the H460 xenograft model (Fig. 2D)]. Nevertheless, in the hollow fiber model, heparin octasaccharides significantly inhibited angiogenesis ($P < 0.05$) to the same degree achieved by paclitaxel, and growth retardation studies suggested that octasaccharides were able to slow the growth of H460 lung carcinoma xenografts.

We know from several investigations that species containing ≥ 12 saccharides have the capacity to support the biological activity of FGF-2. Thus, our observations (Fig. 2A and B) that dodecasaccharides supported FGF-2-induced angiogenesis were consistent with previous reports (11, 13). These data have significant implications for trials of low molecular weight heparin in cancer. In a recent randomized trial of low molecular weight heparin given for a year to patients with advanced cancer, there was no survival advantage for the patients receiving the heparin, although retrospective subgroup analysis did identify a possible group that benefited (22). Our data would suggest that, although a component of low molecular weight heparin would inhibit angiogenesis, most of the preparation will contain species that support FGF-2-induced angiogenesis. Indeed, other data have suggested that species that are long enough to perturb coagulation will also bind and in some cases support the activity of VEGF (4). The implication of our data is that further investigation of heparin preparations for the treatment of cancer should focus on the shorter species as the longer compounds support the activity of angiogenic proteins.

Figure 2A to C shows that both octasaccharides and decasaccharides had potent antiangiogenic effects. If we are to develop these compounds for the clinic, we need to select one species for further development. As there are a large number of heparin-binding and heparin-dependent growth factors (2, 23), it is appropriate to consider other ligands. FGF-1 is a potent angiogenic protein that has different heparin-binding properties from FGF-2. In particular, certain data have suggested that octasaccharides may be able to activate FGF-1 (24). Thus, a simple size definition will not be sufficient for optimum saccharide development. One strategy would involve compositional as well as length modifications. For instance, it is clear that the biological activity of FGF-2 is dependent on sequences of heparan sulfate that contain 6-O-sulfated glucosamine

residues (8), and in accordance with this, we showed (Fig. 2B) that de-6-O-sulfated heparin preparations were also antiangiogenic. Thus, we can overcome the differences in activation sequences for FGF-1 and FGF-2 by combining these two observations by generating de-6-O-sulfated heparin oligosaccharides. Nevertheless, one difficulty with this strategy is the requirement for organic chemical synthesis of defined species, and currently, bulk synthesis of this class of compounds has only been achieved for pentasaccharides (25, 26).

The converse issue is also relevant: Is it possible that heparin oligosaccharides will inhibit the endogenous inhibitors of angiogenesis? Although data are limited, structural studies of endostatin suggest that longer fragments of heparan sulfate that are 6-O-sulfated are required for binding (27). Consistent with these results are the growth inhibition effects (Fig. 2D), which suggest that the net effect on tumor growth is negative.

Other than heparin and low molecular weight heparin, several oligosaccharides have entered the clinic, including tecogalan (DS-4152; ref. 15), PI-88 (16, 28), and pentosan polysulfate (29). These studies have revealed that the principal toxicities of this class of compound are overanticoagulation and immune-mediated thrombocytopenia. In our current study, we failed to document any *in vivo* hemorrhagic toxicity associated with oligosaccharide administration and have also shown that octasaccharides (and other species with < 14 residues) do not adversely affect the coagulation cascade at concentrations that are biologically active *in vitro* (11). These data suggest that short oligosaccharides, which inhibit angiogenesis in our models, are unlikely to cause clinically significant overanticoagulation if given at plasma concentrations up to 40 $\mu\text{g/mL}$. We plan to assess this formally in further *in vivo* studies.

The other common heparin-associated toxicity is heparin-induced thrombocytopenia. This occurs because heparin-induced thrombocytopenia antibodies bind to PF4 that has been conformationally altered by heparin. These antibodies are then taken up by the Fc receptors on platelets, leading to thrombocytopenia and an increased risk of thrombosis. However, at least 12 to 14 saccharide residues are needed to form the antigenic complex with PF4. In accordance, the prevalence of heparin-induced thrombocytopenia in patients treated with low molecular weight heparin is less than that seen in patients receiving unfractionated heparin (30) and preliminary data suggest that heparin-induced thrombocytopenia is even less likely to occur in patients treated with the pentasaccharide fondaparinux (31). Critically, however, this means that heparin-induced thrombocytopenia is very unlikely to occur with heparin-based octasaccharides.

In summary, we have shown that heparin octasaccharides have antiangiogenic activity *in vivo* in several models of variable dependence on FGF-2. The lack of effect on coagulation suggests that we should be able to escalate the doses of these compounds significantly beyond those achieved with other oligosaccharides that have reached the clinic. Indeed, the decades of experience that we have with heparin show that these compounds are safe and well tolerated.

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