Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

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Low-Molecular-Weight–Sulfated *Escherichia coli* K5 Polysaccharide Derivatives as Fibroblast Growth Factor Antagonists

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Objective—Low-molecular-weight heparin (LMWH) exerts antitumor activity in clinical trials. The K5 polysaccharide from *Escherichia coli* has the same structure as the heparin precursor. Chemical and enzymatic modifications of K5 polysaccharide lead to the production of biotechnological heparin-like compounds. We investigated the fibroblast growth factor-2 (FGF2) antagonist and antiangiogenic activity of a series of LMW *N,O*-sulfated K5 derivatives.

Methods and Results—Surface plasmon resonance analysis showed that LMW-K5 derivatives bind FGF2, thus inhibiting its interaction with heparin immobilized to a BIAcore sensor chip. Interaction of FGF2 with tyrosine-kinase receptors (FGFRs), heparan sulfate proteoglycans (HSPGs), and $\alpha_v\beta_3$ integrin is required for biological response in endothelial cells. Similar to LMWH, LMW-K5 derivatives abrogate the formation of HSPG/FGF2/FGFR ternary complexes by preventing FGF2-mediated attachment of FGFR1-overexpressing cells to HSPG-bearing cells and inhibit FGF2-mediated endothelial cell proliferation. However, LMW-K5 derivatives, but not LMWH, also inhibit FGF2/$\alpha_v\beta_3$ integrin interaction and consequent FGF2-mediated endothelial cell sprouting in vitro and angiogenesis in vivo in the chick embryo chorioallantoic membrane.

Conclusions—LMW *N,O*-sulfated K5 derivatives affect both HSPG/FGF2/FGFR and FGF2/$\alpha_v\beta_3$ interactions and are endowed with FGF2 antagonist and antiangiogenic activity. These compounds may provide the basis for the design of novel LMW heparin-like angiostatic compounds. (***Arterioscler Thromb Vasc Biol.*** 2005;25:71-76.)

Key Words: angiogenesis ■ endothelium ■ FGF ■ heparin ■ integrin

Heparin is a natural sulfated glycosaminoglycan (GAG) used as anticoagulant and antithrombotic drug.[1] Heparin is heterogeneous in size: unfractionated heparin (UFH) shows an average molecular weight (MW) of 13 000 to 15 000 with chains ranging from 5000 to 30 000. Heparin structure is largely accounted for by regular trisulfated disaccharide sequences made of alternating α-1,4-linked residues of 2-*O*-sulfated L-iduronic acid (IdoA) and *N,6*-disulfated D-glucosamine (GlcN). These sequences are occasionally interrupted by nonsulfated uronic acids and undersulfated hexosamines,[1] including 3-*O*–sulfated GlcNs present in the antithrombin III–binding pentasaccharidic sequence relevant for anticoagulant activity.[2]

Heparin binds to a variety of biologically active polypeptides.[3] This capacity may be exploited to design heparin-derived drugs for pharmacological interventions in a variety of pathologic conditions besides coagulation and thrombosis, including neoplasia. Actually, heparins show antitumor activity in clinical trials[4–6] without affecting the incidence of thrombotic and bleeding complications, thus suggesting a direct effect of heparins on cancer progression. Indeed, heparins may inhibit malignant growth by different mechanisms,[6,7] including the suppression of tumor neovascularization after interaction with angiogenic growth factors (reviewed in Presta et al[8]).

Fibroblast growth factor-2 (FGF2) is a major heparin-binding angiogenic growth factor and a possible target for antiangiogenic therapies.[9] FGF2 exerts its activity on endothelial cells by interacting with tyrosine-kinase receptors (FGFRs)[10] and heparan sulfate proteoglycans (HSPGs),[11] thus forming HSPG/FGF2/FGFR ternary complexes.[12] Heparin competes with HSPGs and FGFRs for the binding to

FGF2.[13] Therefore, synthetic molecules and chemically modified heparins able to interfere with HSPG/FGF2/FGFR interaction may act as angiogenesis inhibitors (reviewed in Presta et al[8]). Also, FGF2 binds to endothelial $\alpha_v\beta_3$ integrin, and this interaction is required for biological response.[14,15] Accordingly, FGF2/$\alpha_v\beta_3$ integrin interaction antagonists are endowed with antiangiogenic activity in vitro and in vivo.[16]

The capsular K5 polysaccharide from *Escherichia coli* has the same structure [$\rightarrow$ 4)-β-D-GlcA-(1 $\rightarrow$ 4)-α-D-GlcNAc-1(1 $\rightarrow$]$_n$ as the heparin precursor *N*-acetyl heparosan[17] in which GlcA is glucuronic acid and GlcNAc is *N*-acetyl-glucosamine. Chemical and enzymatic modifications of the K5 polysaccharide lead to the synthesis of heparin-like compounds[18] endowed with different biological properties, including anticoagulant/antithrombotic,[19,20] antineoplastic,[21] and anti-AIDS[22] activities. Recently, we demonstrated that a highly *N,O*–sulfated K5 derivative [K5-N,OS(H)] binds FGF2 with high affinity and exerts a potent antiangiogenic activity.[23] Thus, K5-N,OS(H) may provide the basis for the design of novel angiostatic compounds with therapeutic implications in different angiogenesis-dependent diseases, including cancer.

Low-molecular-weight heparins (LMWHs) are obtained by chemical or enzymatic depolymerization of UFH. LMWH has favorable pharmacokinetics compared with conventional heparin.[24,25] Relevant to the use of heparins in cancer therapy, LMWH prolongs disease-free survival time and reduces death rate in cancer patients.[6] In this study, we synthesized a series of LMW derivatives of K5-N,OS(H) that were assessed for their FGF2 antagonist activity in vitro and angiostatic capacity in vivo.

Methods

Materials
Recombinant FGF2 was from Pharmacia-Upjohn (Milan, Italy). UFH (average MW=13 700; sulfate/carboxyl ratio [SO_3^-/COO^-]=2.14) was obtained from unfractionated beef mucosa sodium heparin (Laboratori Derivati Organici, Milan, Italy). LMWH (average MW=5000; SO_3^-/COO^-=2.13) was provided by C. Pisano (Sigma-Tau, Pomezia, Italy). K5 polysaccharide (average MW=30 000) was prepared as described.[23] Cyclo(-Arg-Gly-Asp-D-Phe-Val) peptide [c(RGDfV)] and cyclo(-Arg-Ala-Asp-D-Phe-Val) peptide [c(RADfV)] were from Bachem AG.

LMW-K5 Derivatives
Derivatives were generated by nitrous acid depolymerization and subsequent reduction from a single batch of K5-N,OS(H)[23] (for details, please see the online Methods, available at http://atvb.ahajournals.org.). ^{13}C-NMR spectrum analysis, SO_3^-/COO^- analysis, and MW determinations of the different samples were performed as described.[23]

BIAcore Binding Assay
Biotinilated UFH was immobilized (6.0 fmol/mm²) to a streptavidin-activated sensor chip (BIAcore Inc, Piscataway, NJ). Increasing concentrations of FGF2 in 10 mmol/L HEPES, 150 mmol/L NaCl, 3.4 mmol/L EDTA, 0.005% surfactant P20 (pH 7.4), were then injected over the heparin-coated surface for 4 minutes and washed until dissociation was observed. The signal was expressed as resonance units. Also, FGF2 (160 nmol/L) was injected over the heparin-coated sensor chip in the presence of increasing GAG concentrations. A streptavidin-activated sensor chip was used for blank subtraction.

FGF2-Mediated Cell–Cell Adhesion Assay
Chinese hamster ovary (CHO)-K1 cells were seeded at 52 000 cells per cm² in 24-well plates. After 24 hours, cell monolayers were fixed with 3% glutaraldehyde in PBS. Then, A745 CHO *flg*-1A cells, generated by transfection of GAG-deficient A745 CHO cells[26] with the IIIc variant of murine FGFR1 cDNA,[27] were added at 52 000 cells per cm² to CHO-K1 monolayers in serum-free medium plus 10 mmol/L EDTA with no addition or with 30 ng/mL FGF2 in the presence of increasing concentrations of the different GAGs. After 2 hours at 37°C, cells bound to the monolayer were counted under an inverted microscope.[23]

Cell Proliferation Assay
Bovine endothelial GM7373 cells (Human Genetic Mutual Cell Repository, Camden, NJ) were seeded at 70 000 cells per cm² in 24-well plates. After overnight incubation in Eagle's minimal essential medium containing 10% FCS, cells were incubated in medium containing 0.4% FCS and 10 ng/mL FGF2. After 8 hours, increasing concentrations of GAGs were added to cell cultures without changing the medium. Sixteen hours thereafter, cells were trypsinized and counted.[23]

Fibrin Gel Sprouting Assays
FGF2-transfected murine endothelial FGF2-*T*-MAE cell aggregates[28] were prepared on agarose-coated plates and seeded within fibrin gel.[23] Then, culture medium with or without K5 derivatives (100 μg/mL) or cyclic pentapeptides (60 μmol/L) was added. Formation of radially growing cell sprouts was quantified after 24 hours by image analysis of the digitized images using the Image-Pro Plus software (Media Cybernetics). Freshly prepared rat aorta rings[29] were cultured in fibrin gel for 7 days in the presence of the different antagonists. Rings were examined daily and neovessels counted under an inverted microscope.

Cell Adhesion Assay
Polystyrene nontissue culture 96-well plates were coated with 20 μg/mL FGF2, vitronectin, or fibronectin as described.[14] Next, 50 000 GM7373 cells were seeded in the absence or presence of the antagonist. After 2 hours at 37°C, adherent cells were quantified by methylene blue/Azur II staining.[14]

Chick Embryo Chorioallantoic Membrane Assay
At day 8 of incubation, sterilized gelatin sponges (1 mm³; Gelfoam, Upjohn Co) adsorbed with the K5-derivative dissolved in 3 μL of PBS (50 μg/embryo) were implanted on the top of growing chorioallantoic membranes (CAMs).[23] Sponges containing vehicle alone were used as negative controls. CAMs were examined under a stereomicroscope until day 12, and blood vessels around the sponges were counted (10 eggs per group).

Results

Production of LMW-K5 Derivatives
K5 derivatives with reduced size were prepared by controlled chemical depolymerization of K5-N,OS(H) (SO_3^-/COO^-=3.87; average MW of 20 700). Three LMW compounds [LMW-K5(A), LMW-K5(B), and LMW-K5(C) with MW equal to 6000, 5000, and 4200, respectively] and 1 derivative with an intermediate MW of 11 000 (IMW-K5) were obtained. As for K5-N,OS(H),[23] the compounds carry 1 *N*-sulfated group and 1 6-*O*–sulfated group in all GlcN residues, with 70% of their sequence being represented by GlcA2,3SO_3^--GlcNSO$_3^-$,6SO_3^- disaccharide units (Figure I, available online at http://atvb.ahajournals.org).
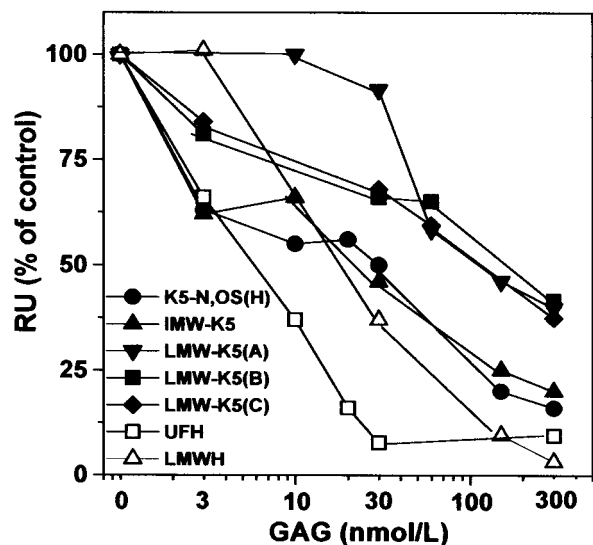
Figure 1. Surface plasmon resonance analysis of LMW-K5 derivative/FGF2 interaction. FGF2 (160 nmol/L) was injected over a heparin-coated BIAcore sensor chip in the presence of increasing concentrations of the different GAGs. The response was recorded at the end of injection and plotted as a function of the GAG concentration.

Binding of LMW-K5 Derivatives to FGF2

LMW-K5 derivatives were evaluated for the capacity to interact with FGF2 by preventing its binding to biotinilated heparin immobilized onto a streptavidin-activated BIAcore sensor chip. FGF2 binds to the heparin-coated sensor chip, but not to the streptavidin-activated sensor chip, in a dose-dependent manner (Figure II, available online at http://atvb.ahajournals.org). An association rate constant equal to 9.0×10^3 M^{-1} s^{-1} and a dissociation rate constant equal to 3.8×10^{-4} s^{-1} characterize the interaction that occurs with high affinity (K_d=42.5 nmol/L), consistent with previous determinations.[30,31]

On this basis, increasing concentrations of LMW-K5 derivatives or UFH were preincubated with FGF2 and then injected onto the heparin-coated sensor chip. All the compounds caused a dose-dependent inhibition of FGF2/heparin interaction (Figure 1). LMW-K5(A), LMW-K5(B), and LMW-K5(C) showed a similar potency (ID_{50}=100 to 120 nmol/L), 4 to 5$\times$ lower than that observed for K5-N,OS(H) and IMW-K5. These differences were, however, abolished when the concentration of the different compounds was expressed on a weight basis (ID_{50} values ranging from 0.3 μg/mL to 0.6 μg/mL). When compared with K5 derivatives, UFH (MW of 13 700) appeared to be a more active competitor (ID_{50}=6.0 nmol/L, corresponding to 0.08 μg/mL). Finally, a LMWH preparation (MW of 5000) inhibited the binding of FGF2 to immobilized heparin with a similar potency (ID_{50}=20 nmol/L, corresponding to 0.1 μg/mL; Figure 1). No effect was instead exerted by unmodified K5 (not shown).

Effect of LMW-K5 Derivatives on HSPG/FGF2/FGFR Ternary Complex

FGF2 mediates cell–cell attachment by linking FGFRs and HSPGs on neighboring cells through the formation of HSPG/FGF2/FGFR ternary complexes.[32] Indeed, HSPG-deficient
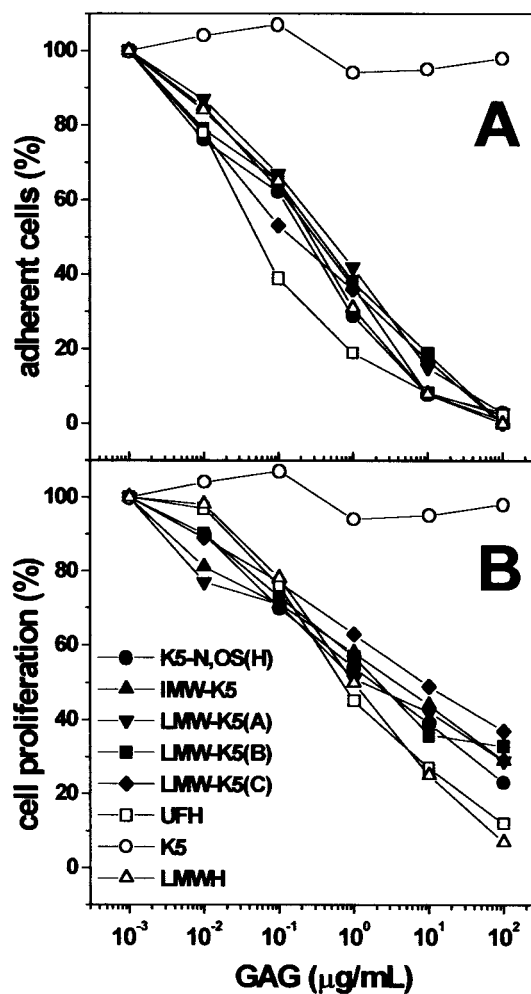

Figure 2. Effect of LMW-K5 derivatives on FGF2-mediated cell–cell adhesion and endothelial cell proliferation. A, HSPG-deficient FGFR1 transfectants were added to CHO monolayers in the presence of 30 ng/mL FGF2 and increasing concentrations of the different GAGs. After 2 hours at 37°C, adherent cells were counted. B, The different GAGs were tested for the capacity to inhibit FGF2-mediated proliferation in GM7373 cells. Data are expressed as percentage of the proliferation measured in the absence of any competitor. In both A and B, experiments were performed in triplicate and repeated 3$\times$ with similar results.

FGFR1-transfected A745-CHO *flg*-1A cells adhere to a monolayer of HSPG-bearing CHO-K1 cells when incubated in the presence of 30 ng/mL FGF2 but not in the absence of the growth factor.[27] K5-N,OS(H) inhibits FGF2-mediated cell–cell attachment in this model.[23]

When tested under the same experimental conditions, all the LMW-K5 compounds and IMW-K5 hamper the formation of the HSPG/FGF2/FGFR ternary complex with an activity equal to that shown by the parent compound K5-N,OS(H) and LMWH (ID_{50}=0.3 μg/mL) and only slightly less potent than that exerted by UFH (ID_{50}=0.1 μg/mL). Unmodified K5 was ineffective (Figure 2A).

Effect of LMW-K5 Derivatives on Endothelial Cell Proliferation and Sprouting

To evaluate a possible angiosuppressive activity of LMW-K5 derivatives, we evaluated their capacity to affect FGF2-
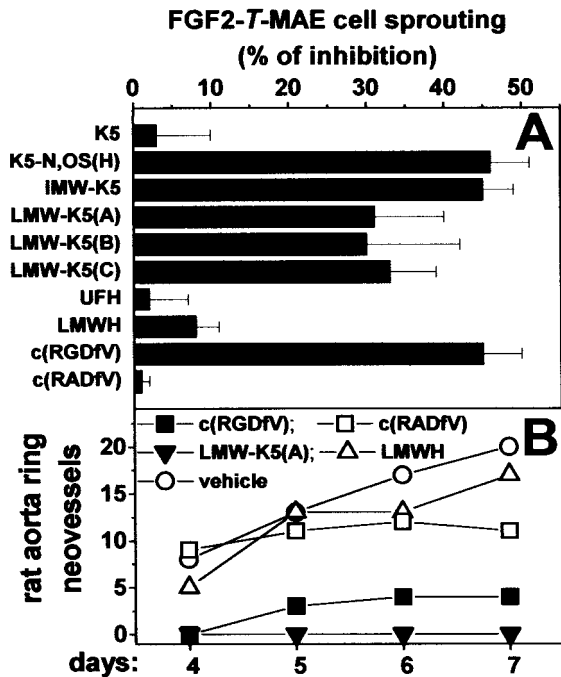
Figure 3. Effect of LMW-K5 derivatives on endothelial cell sprouting. A, FGF2-*T*-MAE cell aggregates were seeded in fibrin gel in the presence of the different GAGs (100 μg/mL) or cyclic peptides (60 μmol/L). After 24 hours, sprouting was evaluated by computerized image analysis. Data are the mean±SEM of 3 experiments in triplicate. B, Rat aorta rings were embedded in fibrin gel in the presence of vehicle or of LMW-K5(A), LMWH (both at 100 μg/mL), or cyclic peptides (60 μmol/L). Neovessels that sprout out of the rings were counted during the next 7 days. Data represent 4 rings per experimental condition. Similar results were obtained in a second independent experiment.

mediated cell proliferation in bovine endothelial GM7373 cells. Similar to heparin and LMWH, LMW-K5 derivatives and IMW-K5 inhibit endothelial cell proliferation (ID$_{50}$=1 to 3 μg/mL). No inhibition was instead exerted by unmodified K5 (Figure 2B).

The capacity of K5 derivatives to affect angiogenesis was investigated further by an in vitro sprout formation assay. In this assay, FGF2-transfected endothelial FGF2-*T*-MAE cell aggregates are embedded into a fibrin gel in which they form solid cell sprouts after 1 to 2 days in culture.[33] As shown in Figure 3A (see also Figure III, available online at http://atvb.ahajournals.org), K5 derivatives exert a significant inhibitory effect on FGF2-*T*-MAE cell sprouting, whereas unmodified K5, UFH, and LMWH are ineffective. Also, endothelial cell sprouting was suppressed by the $\alpha_v\beta_3$ integrin antagonist cyclic pentapeptide c(RGDfV)[34] but not by the control peptide c(RADfV; Figure 3A).

Accordingly, LMW-K5(A) and c(RGDfV), but not LMWH and c(RADfV), inhibit endothelial cell sprouting in an ex vivo assay in which new capillary-like structures originate spontaneously from the endothelium of fibrin-embedded rat aorta rings[29] (Figure 3B and Figure III).

Effect of LMW-K5 Derivatives on FGF2/$\alpha_v\beta_3$ Integrin Interaction

Integrins, including $\alpha_v\beta_3$, play an important role in angiogenesis.[35] Also, FGF2 binds $\alpha_v\beta_3$, and this interaction is required
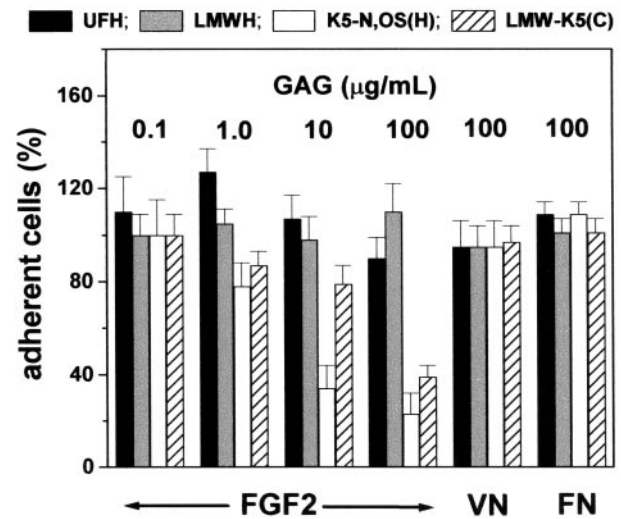

Figure 4. Effect of LMW-K5 derivatives on FGF2/$\alpha_v\beta_3$ interaction. GM7373 cells were seeded on plastic coated with FGF2, fibronectin (FN), or vitronectin (VN) in the absence or presence of UFH, LMWH, K5-N,OS(H), or LMW-K5(C). Adherent cells were counted after 2 hours.

for endothelial cell response to the growth factor.[14–16] The capacity of K5 derivatives to inhibit endothelial cell sprouting similar to the integrin antagonist c(RGDfV) prompted us to assess the possibility that K5 derivatives may affect FGF2/$\alpha_v\beta_3$ interaction. Endothelial cells adhere and spread on FGF2-coated plastic through FGF2/$\alpha_v\beta_3$ interaction.[14,15] Both K5-N,OS(H) and LMW-K5(C) prevent GM7373 cell adhesion to immobilized FGF2, whereas UFH and LMWH are ineffective (Figure 4). Similar results were obtained with LMW-K5(A) and the integrin antagonist c(RGDfV), whereas control c(RADfV) was ineffective (data not shown). The effect of K5 derivatives on FGF2/$\alpha_v\beta_3$-mediated endothelial cell adhesion was specific, because K5-N,OS(H) and LMW-K5(C) did not inhibit cell adhesion to the prototypic integrin ligands vitronectin and fibronectin.

Effect of LMW-K5 Derivatives on Chick Embryo CAM Angiogenesis

Various FGF2 antagonists, including heparin-like compounds and $\alpha_v\beta_3$ antagonists, exert a significant antiangiogenic activity in the chick embryo CAM.[16,23,36,37] When delivered on the top of the CAM through a gelatin sponge implant, all the K5 derivatives inhibited blood vessel growth. This was observed in 70% of the embryos treated with K5-N,OS(H), in 80% of those treated with LMW-K5(A) or LMW-K5(B), and in 90% of the embryos treated with LMW-K5(C). Accordingly, the number of blood vessels surrounding the implants decreased rapidly during incubation with K5-N,OS(H) or with the different LMW-K5 compounds compared with the physiological increase in vascularization observed in vehicle-treated embryos (Figure 5 and Figure III). In agreement with previous observations, unmodified K5 and heparin did not affect CAM neovascularization.[23,36,37]

Discussion

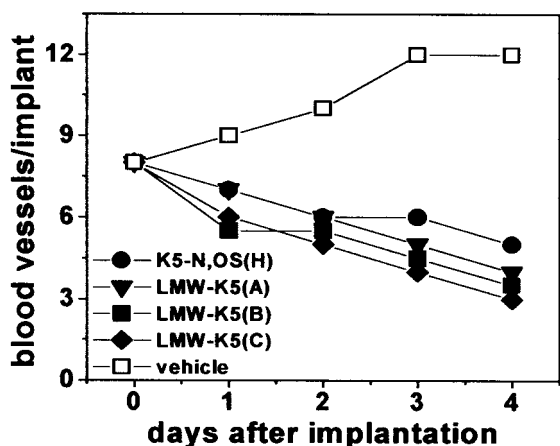Here, a series of LMW-K5 compounds (MW ranging from 6000 to 4200) obtained by controlled chemical depolymer-

Figure 5. Effect of LMW-K5 derivatives on chick embryo CAM vascularization. Gelatin sponges adsorbed with vehicle or K5 derivatives (all at 50 μg per embryo) were implanted on the top of CAMs at day 8. CAMs were examined daily, and blood vessels around the sponges were counted (n=10).

ization of a high MW N,O-sulfated K5 derivative[23] exert a potent FGF2 antagonist activity in vitro and show antiangiogenic capacity in vivo. Thus, as few as 8 monosaccharide units [as in LMW-K5(C)] are sufficient to confer to K5-N,OS(H) derivatives a full angiostatic capacity.

The minimal FGF2-binding sequence in heparan sulfate is a pentasaccharide that contains the disaccharide units IdoA2SO$_3^-$-GlcNSO$_3^-$ or IdoA2SO$_3^-$-GlcNSO$_3^-$,6SO$_3^-$.[38] K5 derivatives can be chemically and enzymatically modified to reproducible structures in which sulfated groups and GlcA/IdoA residues are distributed along the GAG chain in a statistically homogenous manner.[18,39–41] Surface plasmon resonance analysis demonstrates that free K5-N,OS(H), IMW-K5, and all LMW-K5 derivatives ($\approx$70% of their sequence represented by GlcA2,3SO$_3^-$-GlcNSO$_3^-$,6SO$_3^-$ disaccharide units) compete with immobilized heparin for the binding to FGF2 with similar potency and only slightly less efficiently than free UFH or LMWH when GAG chain concentration was expressed on a weight basis. This is in keeping with the hypothesis that more FGF2 molecules bind to a single heparin chain and that the stoichiometry of FGF2/heparin complexes depends on the size of the GAG. A single UFH or LMWH chain binds 6 or 2 FGF2 molecules, respectively, both heparin types giving a heparin mass of 2300 per FGF2-binding site.[42] Similarly, it is possible to hypothesize that LMW-K5 chains can bind 2 to 3 FGF2 molecules per complex and that longer IMW-K5 and K5-N,OS(H) chains will bind a proportionally higher number of growth factor molecules.

FGF2 interaction with nonsignaling HSPGs is required for the binding to FGFRs. FGFR occupancy will then lead to multiple biological responses in cultured endothelial cells and eventually to in vivo neovascularization.[11] Similar to K5-N,OS(H) and IMW-K5, LMW-K5 derivatives abrogate FGF2-mediated attachment of FGFR1-expressing cells to neighboring HSPG-bearing cells, thus indicating their ability to prevent the formation of the HSPG/FGF2/FGFR ternary complex.[23] Synthetic molecules and chemically modified heparins that interfere with HSPG/FGF2/FGFR interaction

act as angiogenesis inhibitors (reviewed in Presta et al[8]). Accordingly, K5-N,OS(H) derivatives suppress neovascularization in the chick embryo CAM.

Similar to K5 derivatives, heparin binds FGF2 and prevents HSPG/FGF2/FGFR interaction and FGF2-driven endothelial cell proliferation in vitro. However, at variance with K5 derivatives, heparin does not affect endothelial cell sprouting in vitro and angiogenesis in vivo. This apparent discrepancy may be explained by the observation that K5 derivatives, but not heparin, also prevent the interaction of FGF2 with $\alpha_v\beta_3$ integrin. $\alpha_v\beta_3$ plays an important role in angiogenesis,[35] and FGF2/$\alpha_v\beta_3$ interaction is required for endothelial cell response to the growth factor.[14–16] Also, numerous observations point to an intimate cross-talk between integrin-mediated intracellular signaling and angiogenic growth factor tyrosine kinase receptor activation.[43] Accordingly, $\alpha_v\beta_3$ represents a target for angiostatic compounds.[44] Thus, the capacity of K5 derivatives to interfere with both HSPG/FGF2/FGFR and FGF2/$\alpha_v\beta_3$ interactions results in a potent angiostatic activity.

K5 derivatives do not inhibit integrin-mediated endothelial cell adhesion to vitronectin (a prototypic $\alpha_v\beta_3$ ligand) or fibronectin (a prototypic $\alpha_5\beta_1$ ligand), indicating that the observed FGF2/$\alpha_v\beta_3$ antagonist activity is because of the capacity of these compounds to bind the growth factor rather than the integrin molecule. The characterization of the structural requirements for the FGF2/$\alpha_v\beta_3$ antagonist activity of K5 derivatives deserves further investigation.

Both thrombotic and hemorrhagic complications have been reported in cancer patients undergoing antiangiogenic therapy (discussed in Daly et al[45]). The occurrence of these life-threatening complications suggests that the use of antiangiogenic compounds endowed with anticoagulant activity or in association with anticoagulants should be approached with caution. Relevant to this point, K5-N,OS(H) and its LMW derivatives are endowed with negligible anticoagulant activity (P.O. and G. Z., unpublished data, 2003). LMW-K5 compounds may therefore provide the basis for the design of novel LMW angiostatic compounds with therapeutic implications in angiogenesis-dependent diseases, including cancer.

Acknowledgments

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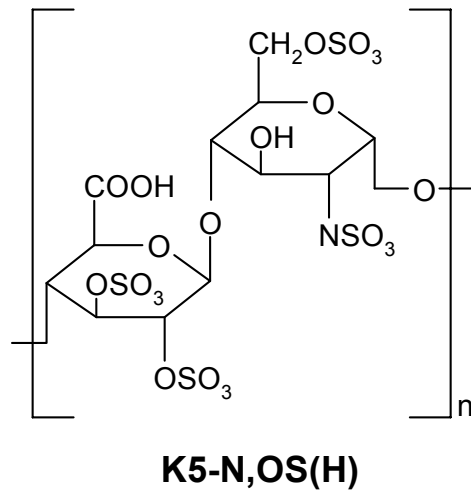
K5-N,OS(H)

Figure I. Schematic structure of K5-N,OS(H) derivatives. 70% of the saccharide sequence in K5-N,OS(H) and its LMW derivatives is represented by $GlcA2,3SO_3^- - GlcNSO_3^-,6SO_3^-$ disaccharide units.

Online publication data Figure I.

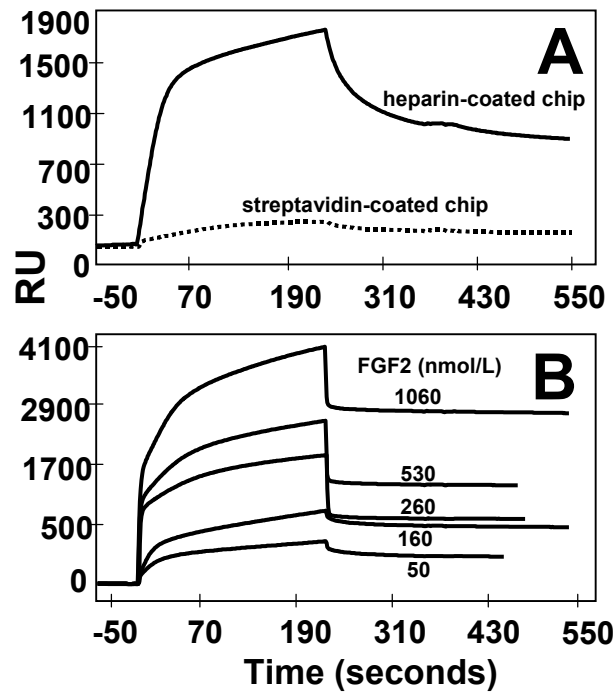**Figure II. Surface plasmon resonance analysis of FGF2/heparin interaction. A)** FGF2 (260 nmol/L) was injected over streptavidin-activated (dotted line) or heparin-coated (straight line) BIAcore sensorchips. The response (in RU) was recorded as a function of time. **B)** Sensogram overlay showing the binding of increasing amounts of FGF2 (1060, 530, 260, 160, and 50 nmol/L from top to bottom) to immobilized heparin.
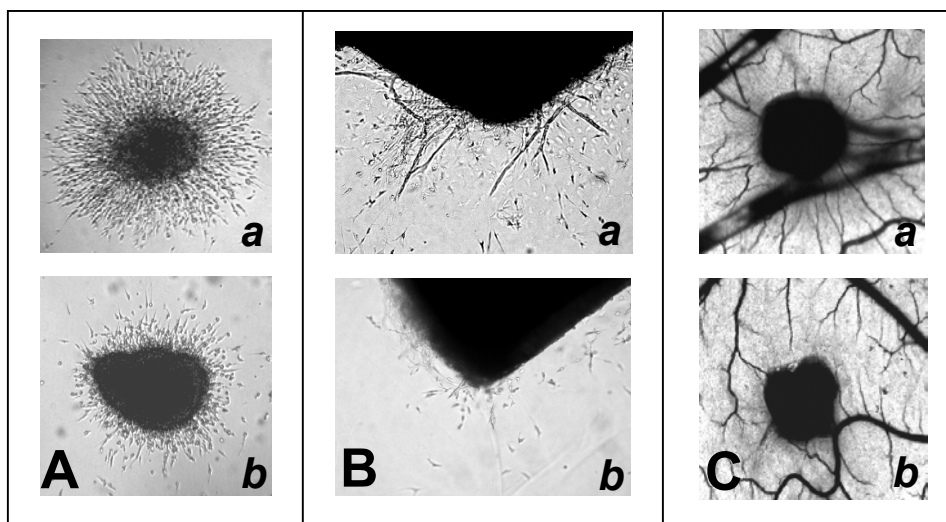
Figure III. Effect of LMW-K5 derivatives on endothelial cell sprouting and angiogenesis. FGF2-*T*-MAE cell aggregates (A) or rat aortic rings (B) were seeded in fibrin gel in the presence of vehicle (*a*) or 100 μg/mL LMW-K5(A) (*b*). Endothelial sprouts were photographed under an inverted microscope after 24 hours (A) or 7 days (B). C) CAMs were treated with vehicle (*a*) or 50 μg LMW-K5(A) (*b*) at day 8 of incubation and photographed at day 12. Note the inhibitory effect exerted on endothelial cell sprouting and angiogenesis by LMW-K5(A). Original magnifications: x40 (A); x100 (B); x20 (C).

Online publication data Figure III.

Synthesis of LMW-K5 derivatives.

Derivatives were generated by nitrous acid depolymerization and subsequent reduction from a single batch of K5-N,OS(H) [average MW = 20,700; $SO_3^-/COO^- = 3.87$] [1]. To this purpose, 20 mL aliquots of an ice-cooled water solution containing 100 mg of K5-N,OS(H) were added with different volumes (from 4.0 to 11.5 mL) of 0.2 % sodium nitrite in water to obtain compounds with different final MW. The pH was adjusted to 2.0 with HCl, samples were kept for 30 minutes at 4°C under gentle stirring, then the pH was brought to pH 7.0 with NaOH. Next, 5 mL of 0.5% sodium borohydride in water were added and samples maintained at room temperature for 4 hours. Then, the pH was brought to 5.0 with HCl and back to 7.0-7.2 with NaOH after 30 minutes. Samples were concentrated, precipitated twice with acetone, and dried in a vacuum oven. ^{13}C-NMR spectrum analysis, SO_3^-/COO^- analysis, and MW determinations of the different samples were performed as described [1].

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