Published in final edited form as:

J Cell Biochem. 2010 October 1; 111(2): 461–468. doi:10.1002/jcb.22727.

Multiple Mechanisms for Exogenous Heparin Modulation of Vascular Endothelial Growth Factor Activity

Errol Wijelath^{1,*}, Mayumi Namekata¹, Jacqueline Murray¹, Mai Furuyashiki², Siyuan Zhang³, Daniel Coan¹, Masahiro Wakao⁴, Robert B. Harris⁵, Yasuo Suda^{2,4}, Lianchun Wang³, and Michael Sobel¹

¹Department of Surgery, Division of Vascular Surgery, VA Puget Sound Health Care System and the University of Washington School of Medicine, Seattle, Washington

²SUDx-Biotec Corporation, Kobe, Japan

³Department of Biochemistry and Molecular Biology, Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia

⁴Department of Nanostructure and Advanced Materials, Graduate School of Science and Engineering, Kagoshima University, Kagoshima, Japan

⁵Commonwealth Biotechnologies, Inc., Richmond, Virginia

Abstract

Heparin and heparin-like molecules are known to modulate the cellular responses to vascular endothelial growth factor-A (VEGF-A). In this study, we investigated the likely mechanisms for heparin's influence on the biological activity of VEGF-A. Previous studies have shown that exogenous heparin's effects on the biological activity of VEGF-A are many and varied, in part due to the endogenous cell-surface heparan sulfates. To circumvent this problem, we used mutant endothelial cells lacking cell-surface heparan sulfates. We showed that VEGF-induced cellular responses are dependent in part on the presence of the heparan sulfates, and that exogenous heparin significantly augments VEGF's cellular effects especially when endogenous heparan sulfates are absent. Exogenous heparin was also found to play a cross-bridging role between VEGF-A₁₆₅ and putative heparin-binding sites within its cognate receptor, VEGFR2 when they were examined in isolation. The cross-bridging appears to be more dependent on molecular weight than on a specific heparin structure. This was confirmed by surface plasmon resonance binding studies using sugar chips immobilized with defined oligosaccharide structures, which showed that VEGF-A₁₆₅ binds to a relatively broad range of sulfated glycosaminoglycan structures. Finally, studies of the far-UV circular dichroism spectra of VEGF-A₁₆₅ showed that heparin can also modulate the conformation and secondary structure of the protein.

Keywords

VEGF-A; VEGFR2; HEPARIN; ENDOTHELIAL CELL; Ndst

Vascular endothelial growth factor-A (VEGF-A), a secreted glycoprotein, plays a key role in regulating both normal and pathological angiogenic processes [Dvorak, 2005]. The importance of VEGF-A in vascular development was demonstrated in mouse models

showing that loss of even a single VEGF-A allele results in lethal vascular defects [Carmeliet et al., 1996]. The main function of VEGF-A is to promote endothelial cell proliferation, migration and survival [Ferrara et al., 2003]. The most abundant spliced variant form of VEGF-A is VEGF-A₁₆₅, which contains a heparin binding domain encoded by exons 6 and 7 [Tischer et al., 1991]. Heparins and heparan sulfates are known to have pleiomorphic effects on the biological actions of VEGF-A [Neufeld et al., 1999]. However, the elucidation of heparin's effects and mechanisms of actions are complicated by the multiplicity of potential heparin-binding partners involved. Not only does VEGF-A₁₆₅ bind heparin, but so do its principal receptor, VEGFR2, as well as VEGFR1 and neuropilin, a co-receptor for VEGF-A [Dougher et al., 1997; Soker et al., 1998; Park and Lee, 1999]. Furthermore, endothelial cells present other heparin-binding sites on their cell surface, including the heparan sulfate proteoglycans (HSPG) [Neufeld et al., 1999]. Exogenous heparin potentiates the binding of VEGF-A to endothelial cells and promotes endothelial cell proliferation, migration and tube formation [Gitay-Goren et al., 1992; Ono et al., 1999; Ashikari-Hada et al., 2005; Lake et al., 2006]. However, most studies of this phenomenon have employed chlorate or heparinase treatments of endothelial cells, which may have unforeseen effects on cellular responses. This present study was undertaken to elucidate the range of influences of heparin on VEGF-A activity, beginning with an endothelial cell line that constitutively lacks sulfation of the cell-surface heparan sulfates, continuing with studies of heparin modulation of VEGF-receptor binding in cell-free systems, and concluding with an examination of the influence of glycosaminoglycan structure on VEGF-A binding and protein conformation.

MATERIALS AND METHODS

VEGF PROTEIN

VEGF-A₁₆₅ was a gift from Genetech. VEGF-A₁₂₁ was expressed in *Pichia pastoris* as described by the manufacturer (Invitrogen).

HEPARINS

Throughout our experiments we employed a standard unfractionated porcine mucosal heparin (molecular weight ~15,000 Da) that has been thoroughly characterized (Celsus, Inc.). For SPR experiments, low molecular weight heparins (LMWH) were prepared by periodate/alkali treatment of a comparable porcine heparin (Nacalai Tesque, Kyoto, Japan), and by fractionation according to their molecular weight as we have previously described [Suda et al., 1993]. For circular dichroism, a LMWH ~5,000 Da was purchased from Calbiochem and a high molecular weight heparin of ~21,000 Da was prepared, as previously described, by affinity fractionation of the standard unfractionated heparin, using a heparin-binding peptide [Poletti et al., 1997].

ENDOTHELIAL CELL LINES

Ndst2^{-/-} and its daughter Ndst1^{-/-} Ndst2^{-/-} mouse lung endothelial cells were generated as previously described [Wang et al., 2005]. In this current study, we utilized these two mutant endothelial cell lines and also a newly generated Ndst1^{+/+}Ndst2^{+/+} line as wild-type control which was similarly derived from wild-type mice. All three endothelial cell lines were derived from fully backcrossed C57Bl/6J mice and possess the same genetic background.

FLOW CYTOMETRY ANALYSIS OF GLYCOSAMINOGLYCAN AND GROWTH FACTOR BINDING TO ENDOTHELIAL CELLS LACKING NDST ENZYMES

Endothelial cells (80% confluent) were harvested with 2 mM EDTA containing 0.1% BSA in PBS (PBS-B-E buffer) and incubated with mouse IgM type anti-CS antibody (1:200,

Sigma) or anti-heparan sulfate antibody (10E4, 1:500, Seikagaku) respectively for 1 h with shaking at 4°C. Normal mouse IgM staining served as background control. For basic fibroblast growth factor (FGF-2) binding studies, harvested endothelial cells were incubated with 0.6 µg/ml biotinylated-FGF-2 in PBS-B-E buffer on ice for 30 min. To detect cell surface VEGF binding, endothelial cells were incubated for 1 h at 4°C with recombinant human biotinylated VEGF₁₆₅ (1.1 µg/ml in PBS; R&D Systems). As controls, the same cells were incubated with an irrelevant biotinylated protein (soybean trypsin inhibitor) (Invitrogen; 8 µg/ml for 45 min at 4°C). Cells were then washed with PBS-B-E buffer and incubated with FITC-conjugated goat anti-mouse IgM (for anti-HS and anti-CS antibody, 1:1000, Invitrogen) for 1 h or with FITC-streptavidin (1:1,000, Pierce) for 15 min on ice. Thereafter, the cells were washed twice with PBS-B-E buffer and analyzed by Cell Lab Quanta SC flow cytometer (Beckman Coulter). Twenty thousand cell counts were collected and analyzed by the FlowJo software (Tree Star, Inc.).

MEASUREMENT OF ERK AND VEGFR2 PHOSPHORYLATION

Preliminary experiments established that 5 ng/ml was the concentration for VEGF-A that activated Erk and VEGFR2 that was below the saturation point. This concentration of VEGF-A allowed us to analyze the change in the phosphorylation state of Erk and VEGFR2 in the presence of heparin. Detection and analysis of Erk and VEGFR2 phosphorylation were performed as described previously [Wijelath et al., 2006]. Signal densities from Western blots were quantified using the NIH Image program, and the results of three or more independent experiments pooled for analysis. Activated Erk and VEGFR2 were expressed as fold increase in phosphorylation over basal (unstimulated) levels.

SURFACE PLASMON RESONANCE BINDING STUDIES

Surface plasmon resonance (SPR) experiments were performed with an SPR-670 M (Moritex, Yokohama, Japan) under the manufacturer's recommended guidelines with slight modifications [Wakao et al., 2008]. Two different approaches were taken. First, heparin modulation of VEGF-165 binding to its receptor VEGFR2 was measured. VEGFR2-Fc protein was immobilized on protein A-coated gold sensor chips. The carboxyl groups immobilized chips were purchased from SUDx-Biotec (Kagoshima, Japan) and were activated for 15 min with a mixture of 0.2 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and 0.05 M *N*-hydroxysuccinimide in 90% dioxane. With continuous monitoring of resonance units, Protein A was immobilized to the activated chip by injecting 2 × 100 µl (100 µg/ml) in PBS pH 6. Excess reactive groups were quenched with 1 M ethanolamine-HCl, pH 8.5. VEGFR2-Fc (10 µg/ml) was then immobilized to protein A by injecting 2 × 100 µl in PBS. A flow cell without immobilized VEGFR2-Fc served as the negative control. For binding studies, VEGF-A was injected into the flow cells in PBS containing 0.05% Tween-20 (PBST) with or without heparin. VEGF-A/heparin mixtures were pre-incubated for 10 min at room-temperature before injection into the flow cell. Sensor chips were regenerated by treating with 1.5 M glycine/NaOH, 3 M NaCl, pH 9.

For the second approach, a novel “sugar chip” technology was used to measure the affinity of VEGF-A for a library of different, defined oligosaccharide structures. The array type sugar chips (GAG-chips) were supplied from SUDx-Biotec as previously described [Suda et al., 2006]. Totally synthetic, defined disaccharide structures derived from heparin and related glycosaminoglycans, as well as positive and negative controls (12 in all) were immobilized on the chips of SPR imaging apparatus (SPRinter, Toyobo, Osaka, Japan). The solvent for the binding experiments was PBST at pH 7.4, run at a flow rate of 15 µl/min at 25°C containing a range of concentrations of VEGF-A (12–400 nM) and heparin. Binding was recorded as resonance units, and the kinetic binding parameters deconvoluted using the manufacturer's software to calculate on- and off-rates and dissociation constants.

CIRCULAR DICHROISM

The far-UV circular dichroism spectra of VEGF-A₁₆₅ and VEGF-A₁₂₁ protein were measured in a Jasco Model 715 Spectropolarimeter equipped with a Jasco Model PTC-348WI Peltier temperature controller. The system is operated by Jasco Hardware Manager (ver. 1.50.00) and data collected by Jasco J-700 Analysis Software (ver. 1.50.01). Protein samples were assayed at 2.78 μ M (0.1 μ g/ml) VEGF-A. A measured volume of 200 μ l of protein was placed in a cell with a 0.1 cm light path length, thermostatted at 20°C. The samples were scanned from 350 to 170 nm at a scan speed of 100 nm/min, and two spectra were accumulated for each sample. Circular dichroism spectra were measured for the protein alone, and after the sequential addition of 0.5 μ M increments of unfractionated heparin. Spectral data were analyzed for secondary structure using Dicroprot (ver 2.4).

RESULTS

HEPARAN SULFATION IS MARKEDLY REDUCED IN NDST1/2-NULL CELLS

To clarify the role of HSPG, particularly their sulfated moieties, we studied the effects of heparins and VEGF-A on mutant strains of mouse endothelial cells that naturally lacked sulfated heparans. We first analyzed the degree of sulfation on Ndst1^{+/+}Ndst2^{+/+} (wild-type), Ndst1^{+/+}Ndst2^{-/-}, and Ndst1^{-/-} Ndst2^{-/-} endothelial cell lines using an anti-heparan sulfate antibody 10E4. Ablation of both Ndst1 and Ndst2 attenuates cell surface 10E4 binding by more than 80% whereas Ndst2 ablation does not alter endothelial cell surface heparan sulfate structure, when compared to Ndst1^{+/+}Ndst2^{+/+} mouse endothelial cells (Fig. 1A). The residual 20% binding of Ndst1^{-/-} Ndst2^{-/-} cells may reflect the binding of the antibody to the *N*-acetylated glucosamine structure of heparan sulfate since the 10E4 antibody recognizes both *N*-sulfated and *N*-acetylated structures of heparan sulfate. To confirm the reduced sulfation of the Ndst1/2^{-/-} endothelial cell surface heparan sulfate, we analyzed the binding of FGF-2 since FGF-2 binding to cell surface heparan sulfate is critically dependent on *N*- and 2-*O*-sulfation. Simultaneous ablation of Ndst1 and Ndst2 attenuated cell surface FGF-2 binding by more than 90% (Fig. 1B). However, Ndst2 ablation alone did not alter endothelial cell surface FGF-2 binding. To determine whether the Ndst1/2 ablation alters cell surface chondroitin sulfates (CS) expression, the mutant cells were stained with anti-CS antibody. Cell surface CS levels were similar among Ndst1^{+/+}Ndst2^{+/+}, Ndst1^{+/+}Ndst2^{-/-} and Ndst1^{-/-} Ndst2^{-/-} endothelial cells, demonstrating that Ndst ablation did not affect CS expression (Fig. 1C). We next examined the binding of VEGF-A on these cell-lines. Cell surface VEGF-A binding analysis showed that, although Ndst2 ablation does not alter endothelial cell surface VEGF-A binding, the simultaneous ablation of Ndst1 and Ndst2 attenuated cell surface VEGF-A binding by about 85% (Fig. 1D). The residual 15% cell surface VEGF-A binding may reflect low affinity binding to VEGFR2. Taken together, these cell surface profiles illustrate that the Ndst1^{+/+}Ndst2^{-/-} endothelial heparan sulfate possesses normal heparan sulfate structure, whereas the Ndst1^{-/-} Ndst2^{-/-} endothelial heparan sulfates have very low or no sulfates. CS expression was unaffected in all cell lines.

ACTIVATION OF ERK AND VEGFR2

As shown above, the Ndst1^{+/+}Ndst2^{-/-} and the Ndst1^{-/-} Ndst2^{-/-} endothelial cell lines are suitable cell-systems to examine the requirement of endogenous heparan sulfates and also the role of exogenous heparin for VEGF-A signaling. Ndst1^{+/+}Ndst2^{+/+}, Ndst1^{+/+}Ndst2^{-/-} and Ndst1^{-/-} Ndst2^{-/-} mouse endothelial cell cultures were simultaneously exposed to VEGF-A (5 ng/ml) and different concentrations of unfractionated heparin (1–50 μ g/ml). Erk phosphorylation was measured after 5 min. Figure 2 illustrates the results of three independent experiments. VEGF alone modestly increased Erk phosphorylation in all three cell lines. Increasing concentrations of exogenous heparin steadily increased Erk

phosphorylation induced by VEGF-A (Fig. 2A). However, cells lacking endogenous heparan sulfates (Ndst1^{-/-} Ndst2^{-/-}) responded much more dramatically to exogenous heparin, far exceeding the increase in Erk induced by heparin/VEGF-A in the Ndst1^{+/+}Ndst2^{+/+} and Ndst1^{+/+}Ndst2^{-/-} cells at 10 µg/ml of heparin only ($P < 0.01$, *t*-test). We then investigated how exogenous heparin would influence VEGF's interaction with its principle cognate receptor, VEGFR2. Figure 2B shows that the pattern of VEGFR2 responses to heparin/VEGF was quite similar to the Erk responses. The Ndst1^{-/-}Ndst2^{-/-} cells deficient in sulfated heparans induced more VEGFR2 phosphorylation in response to heparin (10 µg/ml) than did the Ndst1^{+/+}Ndst2^{+/+} and Ndst1^{+/+}Ndst2^{-/-} cells ($P < 0.05$, *t*-test). Heparin alone had no effect on Erk or VEGFR2 phosphorylation (data not shown).

HEPARIN MODULATION OF VEGF-VEGFR2 RECEPTOR BINDING

Because both VEGF-A₁₆₅ and VEGFR2 bind heparin [Gitay-Goren et al., 1992; Dougher et al., 1997], an additional mechanism for heparin enhancement of VEGF activity is the cross-bridging or stabilization of the VEGF-VEGFR2 complex by heparin. Surface plasmon resonance (SPR) techniques were used to measure VEGF-A binding to VEGFR2 in a cell-free system in real-time. Unfractionated heparin promoted VEGF-A binding to VEGFR2, with maximal binding observed at 1 µg/ml heparin (Fig. 3A). At higher concentrations of heparin (100 µg/ml), binding was inhibited. Low molecular weight heparins (≤5,000 Da) did not enhance VEGF-A binding (Fig. 3B).

STRUCTURAL SPECIFICITY OF HEPARIN-VEGF BINDING BY SPR

To investigate the structural specificity of heparin binding to VEGF-A, we immobilized a library of heparin and other sulfated glycosaminoglycan partial structures onto “array type sugar chips” and measured their kinetic binding of VEGF-165. Table I summarizes these findings. Among the heparin partial structures, those containing glucosamine *N*-sulfates 6-*O*-sulfates (GlcNS6S) had lower K_D , independent of the presence or absence of 2-*O*-sulfation at the iduronic/glucuronic acid residue. For the chondroitin sulfates partial structures, 6-*O*-sulfation of the *N*-acetylgalactosamine (GalNAc) moiety conferred a lower K_D than did 4-*O*-sulfation.

HEPARIN MODULATION OF VEGF SECONDARY STRUCTURE

Circular dichroism spectra were measured from VEGF-A₁₆₅ alone, and after the sequential addition of increments of unfractionated heparin. Control spectra were obtained from heparin alone, as well as heparin with VEGF-A₁₂₁, which does not bind heparin. Sequential additions of heparin reached a maximal effect at 0.5 µM heparin, and no additional conformational change was observed at higher concentrations. Figure 4 shows that the negative peak of VEGF-A₁₆₅ shifted from a minimum at 200.6–209.0 nm. Addition of heparin to VEGF-A₁₂₁ showed no change; the spectra were virtually identical in the absence of heparin and at all concentrations of added heparin (data not shown). The CD spectra of VEGF-A₁₆₅ were also measured in response to a low molecular weight (~5,000) and high molecular weight heparin (~21,000), and the relative changes in secondary structure were compared to the standard unfractionated heparin (~15,000) using an average of the algorithms of Chen, Bolotina, and Chang (Table II). Spectral shifts were essentially complete at a molar ratio of heparin/VEGF-A of 0.54 for the low molecular weight heparin, and 0.36 for the unfractionated and high molecular weight heparins. The secondary structure of VEGF-A alone was predominantly random coil and β-sheet, with lesser elements of β-turn and α-helix respectively. Table II shows that upon the addition of saturating concentrations of heparins, the proportion of α-helix increased significantly in proportion to the molecular weight of the heparin.

DISCUSSION

The biosynthesis of heparan sulfate is a complex process, involving more than 26 enzymes. In particular, *Ndst* catalyzes the deacetylation and *N*-sulfation of the *N*-acetylglucosamine residues in heparan sulfates and plays an essential role in generation of ligand binding sites in mature heparan sulfates. Four related *Ndst* genes are identified (*Ndst1-4*). However in mouse lung endothelial cells only *Ndst1* and *Ndst2* are abundantly expressed. In our previous studies, we derived *Ndst2*^{-/-} and its daughter *Ndst1*^{-/-} *Ndst2*^{-/-} mouse lung endothelial cells [Wang et al., 2005]. In this current study, we employed these endothelial cell lines to further define how heparins modulate VEGF-A biological effects. Cells lacking the *Ndst2* enzyme (*Ndst1*^{+/+}*Ndst2*^{-/-}) still express normal levels of sulfated heparans, and in these experiments they showed a typical response to heparin: enhancement of VEGF-A effects at low concentrations. However, in cells lacking both *Ndst* enzymes (*Ndst1*^{-/-} *Ndst2*^{-/-}) which are devoid of sulfated heparans, exogenous heparin dramatically enhanced VEGF response to VEGFR2 and Erk phosphorylation. One hypothesis is that under normal conditions, heparan sulfate proteoglycans (HSPGs) modulate VEGF-A biological responses in both directions—that is, enhance VEGF signaling but also moderate it. On the positive side, the cell-surface HSPGs are binding sites for VEGF-A, enhancing and stabilizing its binding to its cognate tyrosine receptor kinases. In the absence of sulfated heparans, VEGF-cell interactions would be less effective (as observed especially for VEGFR2, the immediate cellular responder), and exogenous heparin would enhance VEGF-cell binding through cross-bridging effects. On the inhibitory side, engagement of VEGF-A with HSPGs could produce counter-regulatory effects, independently modulating the conventional pathways to VEGF-A activation of the cell. Thus, cells lacking sulfated HSPGs would show even greater enhancement of VEGF-A by exogenous heparin, because they lack the intrinsic counter-regulation initiated by independent HSPG signaling. We recognize that this is a speculative theory, which will require more extensive, focused experiments to confirm or deny it. However, one abundant family of HSPGs, the syndecans, are known to signal independently through their cytoplasmic domains [Oh et al., 1997], and other studies have shown that HSPG can inhibit transforming growth factor beta and platelet derived growth factor signaling [Koyama et al., 1998; Chen et al., 2006].

This research also examined cell-independent mechanisms for heparin's effects. We found in cell-free systems that heparin enhanced the binding of VEGF-A to VEGFR2. However, the concentration of heparin (1 µg/ml) required for maximal VEGF-A binding to VEGFR2 was 10-fold less when compared to the concentration of heparin (10 µg/ml) required for optimal VEGF-A induced Erk activation and VEGFR2 phosphorylation. One plausible explanation for the discrepancy in the optimal concentrations of heparin required in the two assays is that the binding assays were carried out in a cell-free system so that heparin can bind to only VEGF or VEGFR2. However, the Erk and VEGFR2 phosphorylation assays were carried out in endothelial cell lines. In this system, heparin can bind not only to VEGF-A and VEGFR2 but to other ligands that are on the endothelial cell surface and thus a higher concentration of heparin was needed for promoting optimal VEGF-A responses.

Considering that both the VEGF-A and VEGFR2 bind heparin [Dougher et al., 1997], these data suggest that exogenous heparin may also play a cross-bridging role in supporting and sustaining the engagement of the protein ligand with its receptor. This behavior mirrors the effects of heparin on another growth factor/receptor complex, FGF, in which heparin forms a ternary complex with the ligand and receptor [Pellegrini et al., 2000]. In both cases, receptor dimerization contributes to signaling [Li et al., 2008], although unlike FGF, VEGF already exists as a natural dimer. Thus, the current binding studies strongly suggest that the biophysical actions of heparins in FGF-receptor interactions may also apply to VEGF-receptor interactions.

An additional, cell-independent effect of heparin was documented by circular dichroism studies. Heparin binding to VEGF-A₁₆₅ induced a shift in the protein's negative peak from 200.6 to 209 nm. No change was seen with heparin alone, or when heparin was added to VEGF-A₁₂₁. This heparin-induced spectral shift in VEGF-165 corresponded to an increase in the helix content of the protein which was proportional to the molecular weight of the heparin. Heparin is known to modulate the secondary structure of a number of heparin-binding proteins, including the classic case of antithrombin III [Stone et al., 1982; Tyler-Cross et al., 1996] and FGF [Prestrelski et al., 1992]. In previous work with antithrombin III, we have shown that heparin specifically increases the helical content of that protein [Ferran et al., 1992]. There are also precedents from other proteins to suggest that shifts in helix content induced by heparin may affect the biological function of the protein [Almeida et al., 1999]. However, further functional studies will be needed to confirm this for VEGF. We have used the terms heparin and heparan both of which share the same core disaccharide structure, but differ in the density and distribution of sulfated regions. Heparans are less sulfated overall, but in their areas of higher sulfation they resemble heparin in its type and density of sulfation [Robinson et al., 2006].

The effects of a 2-*O*-sulfation in the uronic acid moiety (glucuronic or iduronic) on VEGF-A binding are very modest, if noticeable at all. For an *N*- and 6-*O*-sulfated structure, the presence or absence of a 2-*O* sulfate makes very little difference K_D (12 vs. 16.5). Likewise, starting with a single *N*-sulfated structure, the addition of a 2-*O* sulfate has only a modest effect on the K_D (66 vs. 47), compared with the dramatic effect of adding a 6-*O* sulfate (66 vs. 12). A similar tendency was shown in the case of the partial structures of chondroitin sulfates. The 2-*O*-sulfation of the glucuronic acid did not affect the K_D (13 vs. 15 or 20), but the 6-*O*-sulfation more dramatically affected the K_D (34 vs. 15). This suggests that the interaction depends on the 6-*O*-sulfated structure more than that of the net charge, or the 2-*O* sulfation. These findings are indeed in keeping with the research of Robinson et al. [2006] and Ono et al. [1999]. Looking across different glycosaminoglycan families there are similarities and differences between the structural determinants. For dermatan and heparan, 6-*O* sulfation of the hexosamine residue appears to be important for binding HGF and VEGF, while 2-*O* sulfation of iduronic residues is not essential [Lyon et al., 1994, 1998]. Lyon et al. has found that for dermatan sulfates, the iduronic residue appears to be quite important, yet not so critical for heparins/heparans. Holmborn et al. [2004] observed that heparan sulfate synthesized by the Ndst1 and Ndst2 double mutant mouse embryonic stem cells is still 6-*O*-sulfated but contains no *N*- and 2-*O*-sulfate groups. Our recent studies observed both Ndst1 and Ndst2 are abundantly expressed in endothelial cells [Wang et al., 2005]. Chemical analysis of heparan sulfate synthesized by the Ndst1 and Ndst2 double mutant endothelial cells showed a similar heparan sulfate structural change as reported for the Ndst1 and Ndst2 double mutant mouse embryonic stem cells. The endothelial heparan sulfate structure alteration caused by the Ndst1 and Ndst2 ablation inhibited FGF-2 and VEGF-A binding, which are known to depend on *N*- and/or 2-*O*-sulfation.

We believe that the sulfation pattern of the glycosaminoglycan may be only one of the important determinants for modulation of the complex processes of VEGF-mediated cellular responses. In fact polymer size, as well as the configuration of sulfated domains may be as important for the pro-angiogenic activities of a heparin as are the unique disaccharide structure and specific sulfation. Robinson et al. [2006] has suggested that a heparan sulfate polysaccharide containing a pair of highly sulfated heparin-like regions separated by an unsulfated region functions as a binary ligand that can form a more stable VEGF-heparin complex. Throughout our studies, heparins of larger polymer chain length were more effective in inducing Erk phosphorylation, in promoting VEGF-VEGFR2 binding, and in inducing a conformational change in the VEGF protein. The molecular weight dependency of these processes was also borne out by the work of Rouet et al. [2005], who developed a

high molecular weight heparin-mimetic polymer that further promoted VEGF activity. Studies by Mamluk et al. [2002] show that heparin can bind neurophilin and increase the affinity of that heparin–VEGF interaction. Therefore polymer size as well as the site-specificity of sulfations may play a role in promoting the binding of VEGF with both VEGFR2 and neurophilin to form a ternary complex with strong signaling capability.

In conclusion, these data show that exogenous heparin enhances VEGF-induced cellular responses much more significantly in cells lacking sulfated HSPGs, compared with cells that are sulfated. A plausible explanation is that while both cell types benefit from the pro-angiogenic stimuli of preformed VEGF-heparin complexes, the unsulfated cells may be free of the negative feedback induced by growth factor binding to signaling HSPGs like syndecans. Heparin appears to play important biophysical roles for VEGF-A. Heparin promotes VEGF-A binding to its receptor, and likely forms a ternary VEGF-heparin-VEGFR2 complex. Heparin also alters the conformation of VEGF-A₁₆₅ protein. The specificity of heparin's actions lies in part in the pattern of sulfation sites, but also appears to depend on the polymer chain length, as one would expect for a cross-bridging role. Our knowledge of VEGF-heparin interactions is still not as far advanced as it is for the FGF system or other growth factors. We are hopeful that this current work will be helpful in refining glycosaminoglycans that can ultimately be employed as modulators of angiogenesis.

Acknowledgments

This research was supported by grants from NIH (RO1HL097182 to M.S. and P41RR005351 to L.W. with James H. Prestegard as the PI) and the Japan Science and Technology Agency to Y.S.

Grant sponsor: NIH; Grant numbers: RO1HL097182, P41RR005351; Grant sponsor: Japan Science and Technology Agency.

REFERENCES

- Almeida PC, Nantes IL, Rizzi CC, Judice WA, Chagas JR, Juliano L, Nader HB, Tersariol IL. Cysteine proteinase activity regulation. A possible role of heparin and heparin-like glycosaminoglycans. *J Biol Chem.* 1999; 274:30433–30438. [PubMed: 10521421]
- Ashikari-Hada S, Habuchi H, Kariya Y, Kimata K. Heparin regulates vascular endothelial growth factor165-dependent mitogenic activity, tube formation, and its receptor phosphorylation of human endothelial cells. Comparison of the effects of heparin and modified heparins. *J Biol Chem.* 2005; 280:31508–31515. [PubMed: 16027124]
- Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoek A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature.* 1996; 380:435–439. [PubMed: 8602241]
- Chen CL, Huang SS, Huang JS. Cellular heparan sulfate negatively modulates transforming growth factor-beta1 (TGF-beta1) responsiveness in epithelial cells. *J Biol Chem.* 2006; 281:11506–11514. [PubMed: 16492675]
- Dougher AM, Wasserstrom H, Torley L, Shridaran L, Westdock P, Hileman RE, Fromm JR, Anderberg R, Lyman S, Linhardt RJ, Kaplan J, Terman BI. Identification of a heparin binding peptide on the extracellular domain of the KDR VEGF receptor. *Growth Factors.* 1997; 14:257–268. [PubMed: 9386990]
- Dvorak HF. Angiogenesis: Update 2005. *J Thromb Haemost.* 2005; 3:1835–1842. [PubMed: 16102050]
- Ferran DS, Sobel M, Harris RB. Design and synthesis of a helix heparin-binding peptide. *Biochemistry.* 1992; 31:5010–5016. [PubMed: 1599927]
- Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med.* 2003; 9:669–676. [PubMed: 12778165]

- Gitay-Goren H, Soker S, Vlodavsky I, Neufeld G. The binding of vascular endothelial growth factor to its receptors is dependent on cell surface-associated heparin-like molecules. *J Biol Chem.* 1992; 267:6093–6098. [PubMed: 1556117]
- Holmborn K, Ledin J, Smeds E, Eriksson I, Kusche-Gullberg M, Kjellen L. Heparan sulfate synthesized by mouse embryonic stem cells deficient in NDST1 and NDST2 is 6-*O*-sulfated but contains no *N*-sulfate groups. *J Biol Chem.* 2004; 279:42355–42358. [PubMed: 15319440]
- Koyama N, Kinsella MG, Wight TN, Hedin U, Clowes AW. Heparan sulfate proteoglycans mediate a potent inhibitory signal for migration of vascular smooth muscle cells. *Circ Res.* 1998; 83:305–313. [PubMed: 9710123]
- Lake AC, Vassy R, Di BM, Lavigne D, Le VC, Perret GY, Letourneur D. Low molecular weight fucoidan increases VEGF165-induced endothelial cell migration by enhancing VEGF165 binding to VEGFR-2 and NRP1. *J Biol Chem.* 2006; 281:37844–37852. [PubMed: 17028197]
- Li X, Claesson-Welsh L, Shibuya M. VEGF receptor signal transduction. *Methods Enzymol.* 2008; 443:261–284. [PubMed: 18772020]
- Lyon M, Deakin JA, Mizuno K, Nakamura T, Gallagher JT. Interaction of hepatocyte growth factor with heparan sulfate. Elucidation of the major heparan sulfate structural determinants. *J Biol Chem.* 1994; 269:11216–11223. [PubMed: 8157651]
- Lyon M, Deakin JA, Rahmoune H, Fernig DG, Nakamura T, Gallagher JT. Hepatocyte growth factor/scatter factor binds with high affinity to dermatan sulfate. *J Biol Chem.* 1998; 273:271–278. [PubMed: 9417075]
- Mamluk R, Gechtman Z, Kutcher ME, Gasiunas N, Gallagher J, Klagsbrun M. Neuropilin-1 binds vascular endothelial growth factor 165, placenta growth factor-2, and heparin via its b1b2 domain. *J Biol Chem.* 2002; 277:24818–24825. [PubMed: 11986311]
- Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J.* 1999; 13:9–22. [PubMed: 9872925]
- Oh ES, Woods A, Couchman JR. Syndecan-4 proteoglycan regulates the distribution and activity of protein kinase C. *J Biol Chem.* 1997; 272:8133–8136. [PubMed: 9079625]
- Ono K, Hattori H, Takeshita S, Kurita A, Ishihara M. Structural features in heparin that interact with VEGF 165 and modulates its biological activity. *Glycobiology.* 1999; 9:705–711. [PubMed: 10362840]
- Park M, Lee ST. The fourth immunoglobulin-like loop in the extracellular domain of FLT-1, a VEGF receptor, includes a major heparin-binding site. *Biochem Biophys Res Commun.* 1999; 264:730–734. [PubMed: 10544000]
- Pellegrini L, Burke DF, von DF Mulloy B, Blundell TL. Crystal structure of fibroblast growth factor receptor ectodomain bound to ligand and heparin. *Nature.* 2000; 407:1029–1034. [PubMed: 11069186]
- Poletti LF, Bird KE, Marques D, Harris RB, Suda Y, Sobel M. Structural aspects of heparin responsible for interactions with von Willebrand factor. *Arterioscler Thromb Vasc Biol.* 1997; 17:925–931. [PubMed: 9157957]
- Prestrelski SJ, Fox GM, Arakawa T. Binding of heparin to basic fibroblast growth factor induces a conformational change. *Arch Biochem Biophys.* 1992; 293:314–319. [PubMed: 1536567]
- Robinson CJ, Mulloy B, Gallagher JT, Stringer SE. VEGF165-binding sites within heparan sulfate encompass two highly sulfated domains and can be liberated by K5 lyase. *J Biol Chem.* 2006; 281:1731–1740. [PubMed: 16258170]
- Rouet V, Hamma-Kourbali Y, Petit E, Panagopoulou P, Katsoris P, Barritault D, Caruelle JP, Courty J. A synthetic glycosaminoglycan mimetic binds vascular endothelial growth factor and modulates angiogenesis. *J Biol Chem.* 2005; 280:32792–32800. [PubMed: 16014624]
- Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell.* 1998; 92:735–745. [PubMed: 9529250]
- Stone AL, Beeler D, Oosta G, Rosenberg RD. Circular dichroism spectroscopy of heparin-antithrombin interactions. *Proc Natl Acad Sci USA.* 1982; 79:7190–7194. [PubMed: 6961402]
- Suda Y, Marques D, Kermode JC, Kusumoto S, Sobel M. Structural characterization of heparins binding domain for human platelets. *Thromb Res.* 1993; 69:501–508. [PubMed: 8503119]

- Suda Y, Arano A, Fukui Y, Koshida S, Wakao M, Nishimura T, Kusumoto S, Sobel M. Immobilization and clustering of structurally defined oligosaccharides for sugar chips: An improved method for surface plasmon resonance analysis of protein-carbohydrate interactions. *Bioconj Chem*. 2006; 17:1125–1135. [PubMed: 16984119]
- Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC, Abraham JA. The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J Biol Chem*. 1991; 266:11947–11954. [PubMed: 1711045]
- Tyler-Cross R, Sobel M, McAdory LE, Harris RB. Structure-function relations of antithrombin III-heparin interactions as assessed by biophysical and biological assays and molecular modelling of peptide pentasaccharide docked complexes. *Arch Biochem Biophys*. 1996; 334:206–213. [PubMed: 8900394]
- Wakao M, Saito A, Ohishi K, Kishimoto Y, Nishimura T, Sobel M, Suda Y. Sugar chips immobilized with synthetic sulfated disaccharides of heparin/heparan sulfate partial structure. *Bioorg Med Chem Lett*. 2008; 18:2499–2504. [PubMed: 18343110]
- Wang L, Fuster M, Sriramaraio P, Esko JD. Endothelial heparan sulfate deficiency impairs L-selectin- and chemokine-mediated neutrophil trafficking during inflammatory responses. *Nat Immunol*. 2005; 6:902–910. [PubMed: 16056228]
- Wijelath ES, Rahman S, Namekata M, Murray J, Nishimura T, Mostafavi-Pour Z, Patel Y, Suda Y, Humphries MJ, Sobel M. Heparin-II domain of fibronectin is a vascular endothelial growth factor-binding domain: Enhancement of VEGF biological activity by a singular growth factor/matrix protein synergism. *Circ Res*. 2006; 99:853–860. [PubMed: 17008606]

Fig. 1. Analysis of cell surface glycosaminoglycan sulfation in mouse endothelial cells lacking the *Ndst* enzymes. Flow cytometry was employed to phenotype the surface expression of sulfated heparans in mouse endothelial cells of these genotypes: *Ndst1*^{+/+}*Ndst2*^{+/+} (wild-type), *Ndst1*^{+/+}*Ndst2*^{-/-} and *Ndst1*^{-/-}*Ndst2*^{-/-}. Cells were exposed to the primary antibody or biotinylated ligand, and then stained with a secondary FITC-conjugated goat anti-mouse IgM or with FITC-streptavidin. In each panel, the gray histograms represent controls (either irrelevant primary isotype antibody, or only the secondary antibody), and the red histograms are cells stained with the antibody of interest. Panel A: Anti-heparan sulfates antibody that is sulfation specific; (B) biotinylated FGF-2; (C) anti-chondroitin sulfates (anti-CS) antibody; (D) biotinylated VEGF-A₁₆₅. Data are representative of three separate experiments.

Fig. 2. VEGF-mediated Erk and VEGFR2 phosphorylation in mouse endothelial cells lacking heparan sulfates. Mouse endothelial cell-lines lacking heparan sulfates (Ndst1^{-/-}Ndst2^{-/-}) and normally sulfated mouse endothelial cell-lines (Ndst1^{+/+}Ndst2^{+/+} and Ndst1^{+/+}Ndst2^{-/-}) were incubated in serum-free MCDB 131 medium for 2 h prior to stimulation with VEGF-A for 5 min. Control cultures (Ctrl) received no VEGF-A or heparin treatment. Remaining cultures were stimulated with VEGF-A (5 ng/ml) with or without heparin at the indicated concentrations. V p Hep 1, V p Hep 10 and V p Hep 50 denotes VEGF-A (5 ng/ml), plus heparin 1 µg/ml, 10 µg/ml and 50 µg/ml respectively. A: Phosphorylated Erk and (B) VEGFR2 levels were measured by Western blotting and quantified as described in the Materials and Methods Section. Values are means ± SEM (n = 3). Statistical significance was determined by the Wilcoxon rank-sum test. In each panel, the Ndst1^{-/-}Ndst2^{-/-} was significantly higher than its indicated comparators.

Fig. 3.

Heparin modulation of VEGF-A binding to VEGFR2. SPR technology was employed to measure VEGF-A binding to immobilized VEGFR2, as described in the Materials and Methods Section. A: Various concentrations of unfractionated heparin (average molecular weight ~15 kDa) mixed with VEGF-A (5 ng/ml) were injected over immobilized VEGFR2-Fc chimera at a flow rate of 15 μ l/min. B: Different molecular weight heparins (1 μ g/ml) mixed with VEGF-A (5 ng/ml) were injected over immobilized chimeric VEGFR2-Fc protein at a flow rate of 15 μ l/min. Data are presented as means $\pm$ SEM (n = 3).

Fig. 4.

Circular dichroism spectra of VEGF-A₁₆₅. Spectral scans were obtained as described in the Materials and Methods Section. Upon the addition of increments of heparin, there was a maximal shift in the negative peak of VEGF from 200.6 to 209 nm, at a heparin concentration of 0.5 μM. Heparin alone showed no shift in the area of interest.

TABLE I

Structural Specificity of VEGF Binding to Defined Glycosaminoglycan Structures

Sugar sequence	Partial structure of	K_D (nM)	$k_a \times 10^3$ ($M^{-1} \text{ sec}^{-1}$)	$k_d \times 10^{-3}$ (sec^{-1})
GlcA β 1-3GalNAc4S β 1-6Glc	Chondroitin sulfates A	34.1	115.6	3.94
GlcA β 1-3GalNAc6S β 1-6Glc	Chondroitin sulfates C	14.7	111.0	1.64
GlcA2S β 1-3GalNAc6S β 1-6Glc	Chondroitin sulfates D	12.6	88.7	1.12
GlcA β 1-3GalNAc4S6S β 1-6Glc	Chondroitin sulfates E	19.6	64.6	1.26
GlcNS α 1-4GlcA β 1-6Glc	Heparin	66.1	62.7	4.15
GlcNS α 1-4IdoA2S α 1-6Glc	Heparin	46.8	68.4	3.20
GlcNS6S α 1-4GlcA β 1-6Glc	Heparin	12.0	88.0	1.05
GlcNS6S α 1-4IdoA2S α 1-6Glc	Heparin	16.5	78.5	1.30
GlcA β 1-6Glc	Control	151.9	73.9	11.23
Gal β 1-4Glc	Control	NB	NB	NB
Glc β 1-6Glc	Control	NB	NB	NB
Unfractionated heparin	Heparin	11.3	49.1	0.56

Surface Plasmon Resonance studies were performed as described in the Materials and Methods Section, using a multi-channel analyzer and a sugar-chip array immobilized with the defined synthetic structures indicated. Dissociation constant (K_D), on-rate (k_a), and off-rate (k_d) were calculated from multiple runs, using the manufacturer's software. NB, no binding.

Changes in the Estimated Percentages of Secondary Structure of VEGF in Response to Heparins

TABLE II

	α -Helix	β -Sheet	β -Turn	Random coil	Total
VEGF alone	5	32	20	43	100
LMWH	9	25	22	44	100
Unfx heparin	15	22	22	41	100
HMWH	33	1	24	42	100

CD spectra were obtained in the absence and presence of saturating concentrations of heparin (0.9 mol/mol LMWH/VEGF, 0.35 mol/mol for unfractionated (unfx) and high molecular weight heparin (HMWH). The percentage of secondary structure was estimated using the average of 3 algorithms, calculated from two independent spectral scans each.