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Article

Potentiation of Fibroblast Growth Factor Activity by Synthetic Heparin Oligosaccharide Glycodendrimers

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SUMMARY

Heparin is a highly sulfated polysaccharide that regulates a variety of cellular processes by interaction with a host of proteins. We report the preparation of synthetic heparin oligosaccharide glycodendrimers and their use as heparin mimetics to regulate heparin-protein interactions. The multivalent display of sugar epitopes mimics the naturally occurring glycans found on cell surfaces and enhances their binding capacity. Binding of the heparin dendrimers to basic fibroblast growth factor (FGF-2) was analyzed using heparin microarray experiments and surface plasmon resonance measurements on gold chips. Heparin-coated dendrimers bind FGF-2 significantly more effectively than monovalent heparin oligosaccharides. Dendrimer 1, which displays multiple copies of the sulfated hexasaccharide ($GlcNSO_3$[6-OSO_3]-IdoA[2-OSO_3])$_3$, was employed to promote FGF-2-mediated mitogen-activated kinase activation, demonstrating the utility of glycodendrimers to modulate heparin-protein interactions.

INTRODUCTION

Heparin, a highly sulfated and heterogeneous polysaccharide, has been the drug of choice in the prevention and treatment of thromboembolic disorders for nearly 70 years [1]. In addition to their antithrombic activity, heparin and the structurally related heparan sulfate are involved in a wide variety of biological processes by interaction with a host of proteins [2–9]. For instance, heparan sulfate present on cell surfaces plays a key role in leukocyte entry into sites of inflammation by interaction with P- and L-selectins and proinflammatory chemokines [10, 11]. The interaction of heparin with growth factors, such as FGF-2, modulates cell proliferation and angiogenesis [12–14]. Consequently, heparin analogs as therapeutic substitutes of the natural product, with a similar or even better pharmacological profile but lacking the disadvantages of heparin, are of considerable interest. The natural biopolymer holds the potential risk of pathogen contamination because it is isolated from mammalian sources. Undesirable, severe side effects associated with heparin include heparin-induced thrombocytopenia and bleeding [15].

Dendrimers are hyperbranched synthetic macromolecules of defined structure and molecular weight that have been evaluated for biomedical applications [16]. Placement of terminal sugar residues on dendrimers creates a multivalent display that mimics cell-surface glycans [17–19]. Multivalent presentation of sugar epitopes on an appropriate macromolecular scaffold increases binding due to the cluster effect and improves carbohydrate-protein interactions [20–23].

Recently, we prepared amine-functionalized heparin oligosaccharides for immobilization on glass slides to produce heparin chips [24–28]. This amine terminus allows for the attachment to dendrimers to create heparin functionalized conjugates of well-defined structure. Anionic dendrimers containing D-glucosamine and D-glucosamine 6-sulfate prevent scar tissue formation after glaucoma filtration surgery in a rabbit model [29]. Dendrimers with cationic end groups are often toxic [29]. Therefore, we decided to use anionic, polyamidoamine (PAMAM) dendrimers to prepare multivalent conjugates of synthetic heparin oligosaccharides. Dendrimer binding to FGF-2 was analyzed using heparin microarrays and surface plasmon resonance (SPR) measurements on gold chips. Heparin-coated dendrimers bind FGF-2 significantly better than monovalent heparin oligosaccharides. Dendrimer **1** (Figure 1), which displays several copies of the sulfated hexasaccharide **6**, promotes FGF-2-mediated activation of mitogen-activated kinase signaling. Therefore, heparin dendrimers may potentially constitute novel therapeutic agents for tissue repair and regeneration.

RESULTS AND DISCUSSION

Preparation of Synthetic Heparin Oligosaccharide Glycodendrimers

Four different synthetic heparin sequences (**6**–**9**) containing an amine group linked to the reducing ends were used

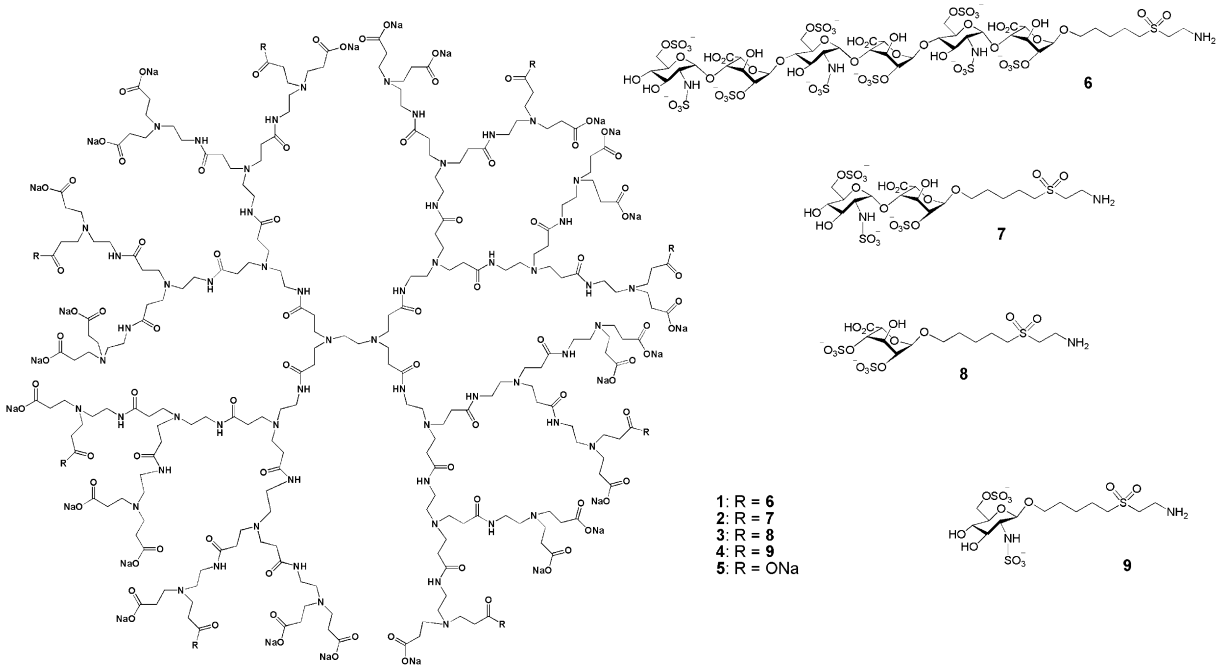

Figure 1. Structure of Polyvalent Dendrimer Conjugates 1–5 and Heparin Oligosaccharides 6–9

to construct heparin glycodendrimers **1**–**4** (Figure 1). Disulfated monosaccharide **9** served as a model to optimize the coupling chemistry. Generation 2.5 PAMAM dendrimer **5**, which contains 32 carboxylic acid groups, was activated with *N*-ethyl-*N*′-(dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) in DMSO. Coupling with **9** in the presence of triethylamine gave, after purification by centrifugal ultrafiltration, the desired glycodendrimer **4**. Sugar conjugation was estimated by ^{1}H NMR in D_2O. Comparison of the signals of sugar protons and protons for the dendritic core indicated that approximately eight sugar residues on each dendrimer were present (25% loading) (Experimental Procedures; see the Supplemental Data available with this article online). Attempts to increase sugar loading by varying reaction time and EDC, NHS, and ligand per carboxylic acid concentrations did not improve conjugation. The steric hindrance or electrostatic repulsion between the anionic dendrimer and the sulfate sugar groups likely prevents higher substitution. Multivalent display and accessibility of sugar epitopes for protein recognition should be suitable at 25% loading. Following this protocol, glycodendrimers **1**, **2**, and **3** were obtained by coupling the PAMAM dendritic core and oligosaccharides **6**–**8**.

Competition Experiments on Heparin Microarrays to Estimate Binding Affinities of Glycodendrimers and FGF-2

FGF-2 is a prototypical member of the fibroblast growth factor (FGF) family and participates in a variety of biological processes including cell proliferation, cell differentiation, and angiogenesis [12, 30]. The heparin binding profile of FGF-2 was recently determined using heparin microarrays [24, 27]. FGF-2 strongly binds hexasaccharide **6**. Disaccharide **7** and monosaccharide **8** are bound less tightly, whereas **9** is not bound at all.

Binding affinities of dendrimers **1**–**3** and FGF-2 were analyzed by determining IC_{50} values [31] of soluble dendrimers with heparin-coated microarrays. The monovalent sugars **6**–**9** were also employed in these experiments. IC_{50} values were defined as concentrations of soluble competitor that inhibit 50% of FGF-2 binding to immobilized heparin. CodeLink glass slides were subdivided into eight different sections using an eight-well hybridization chamber [32]. A deaminated heparin sample (5 kDa) was functionalized with 1,11-diamino-3,6,9-trioxaundecane by reductive amination and printed in each well. Thus, eight different binding experiments can be performed on each slide (Figure 2). A series of FGF-2 (29 nM) mixtures with competitors (0.5 nM to 500 μM) was incubated on the arrays. A solution of FGF-2 without competitor served as control. Preincubation of the protein solution with the competitor for 1 hr prior to hybridization on the glass slide did not influence the results. Bound FGF-2 was detected by incubation with rabbit anti-FGF-2 polyclonal antibody followed by hybridization with fluorescently labeled anti-rabbit secondary antibody [24, 27].

Dendrimer **1** (IC_{50} = 1.4 μM; log IC_{50} = −5.86 ± 0.13) competed more effectively with immobilized heparin than monovalent heparin oligosaccharide **6** (Figure 3). Similarly, IC_{50} values for **2** (43 μM; log IC_{50} = −4.37 ± 0.19) and **3** (165 μM; log IC_{50} = −3.78 ± 0.13) were determined. However, even when using high concentrations of monovalent **7** and **8** (2.5 mM), only negligible inhibition was observed. These observations indicate that dendrimers **2** and **3** are significantly better ligands for FGF-2

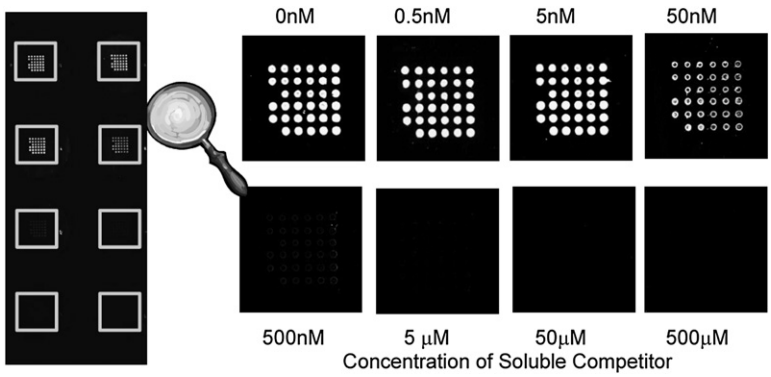

Figure 2. Eight-Well Array for Competition Assays

Amine-functionalized heparin (5 kDa) was printed in each well at two different concentrations (0.5 mM and 1 mM, from top to bottom), in 17 replicates. The wells were incubated with mixtures of FGF-2 and competitor (5 kDa heparin in this figure) and the bound protein was detected by antibody staining using rabbit anti-FGF-2 polyclonal and Alexa Fluor 546-labeled anti-rabbit antibodies. This microarray competition experiment allowed for the estimation of binding affinities of compounds **1**–**9**.

than oligosaccharides **7** and **8**. Still, both glycodendrimers **2** and **3** displayed weaker binding than **1**. Soluble heparin (5 kDa) served as positive control in these competition assays. The IC_{50} value of soluble heparin (28 nM; log IC_{50} = −7.55 ± 0.15) is in good accordance with reported data using an optical biosensor and 12.5 kDa heparin [33] (Supplemental Data). Simple nonspecific charge-charge interactions can be ruled out because **4**, **5**, and **9** failed to inhibit FGF-heparin binding, even at high concentrations (Supplemental Data). These results are in agreement with the carbohydrate binding profile of FGF-2 [24, 27] and suggest that the multivalent presentation of heparin oligosaccharides on an appropriate macromolecular scaffold amplifies their affinity for heparin-binding proteins.

SPR Experiments to Determine Binding Affinities

The binding results obtained with heparin microarrays were validated by SPR experiments. The characterization

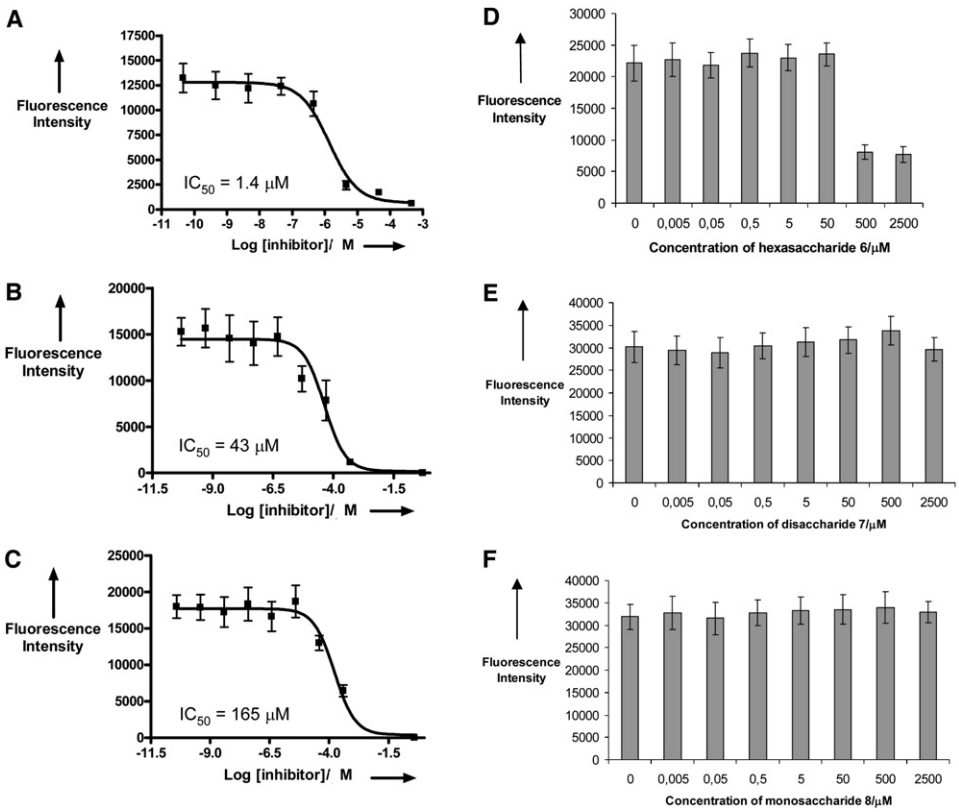

Figure 3. Binding Affinities Using Microarray Experiments

Determination of concentrations of soluble glycodendrimers **1** (A), **2** (B), and **3** (C) to inhibit 50% of FGF-2 binding to immobilized heparin on the carbohydrate microarray (IC_{50} values) and ability of monovalent **6** (D), **7** (E), and **8** (F) to compete with immobilized heparin in FGF-2 binding. For the dendrimers, the concentration is moles of oligosaccharide. Data presented are the average of 17 spots on the same array; errors are the standard deviations for each measurement.

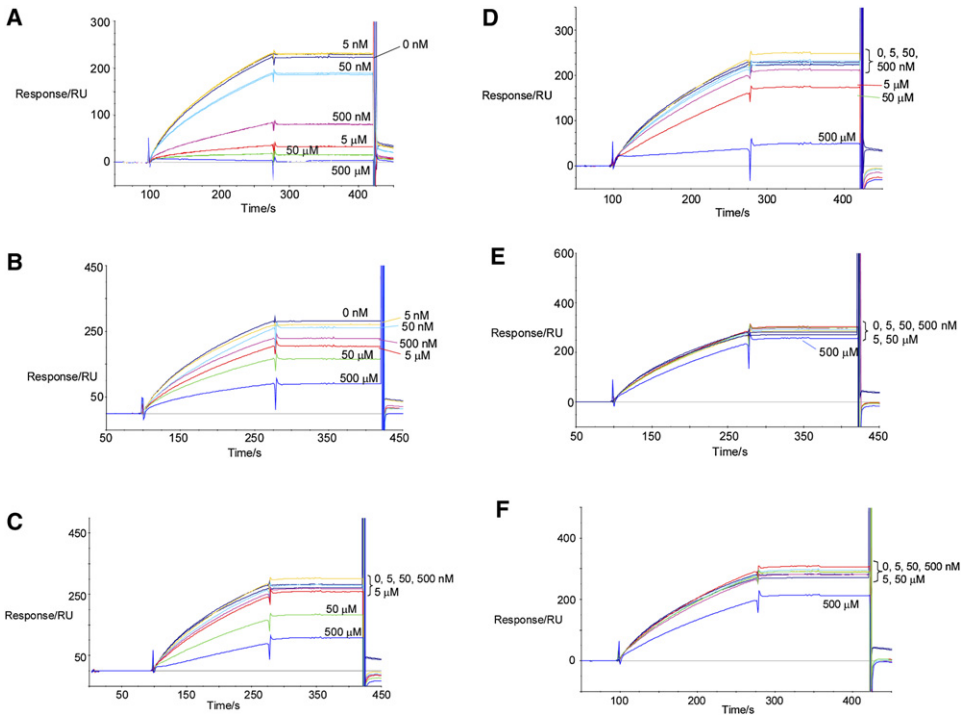

Figure 4. Binding Affinities Using SPR Measurements

SPR curves showing the real-time binding of a series of mixtures of FGF-2 (25 nM) and compounds **1** (A), **2** (B), **3** (C), **6** (D), **7** (E), and **8** (F) (5 nM to 500 μM) to a sensor chip presenting hexasaccharide **6**. For the dendrimers, the concentration is moles of oligosaccharide. RU, response units.

of dendrimer-FGF-2 interactions using an optical biosensor rules out any artifact caused by primary and secondary antibody staining. Moreover, SPR provides additional information on heparin-protein interactions in real time [34]. Inhibition of FGF-2 binding to heparin-like surfaces by heparin dendrimers was measured using a gold chip carrying immobilized **6**.

Biotinylated heparin or heparan sulfate is usually immobilized on a streptavidin-coated sensor chip [35–38]. Synthetic, amine-functionalized heparin oligosaccharides were directly immobilized on the gold surfaces. Hexasaccharide **6** was coupled to an activated CM5 gold chip by using a 1 mM solution of hexadecyltrimethylammonium chloride. The covalent amide bond formation on CM5 chips is favored in a low-ionic strength buffer at a pH below the isoelectric point of the molecule that is immobilized [39]. The ligand is concentrated on the chip surface by electrostatic attraction between the positively charged ligand and the negatively charged carboxyl groups of the chip. Immobilization of highly sulfated heparin oligosaccharides (e.g., **6**) by amine coupling is difficult, as the presence of negatively charged sulfate groups prevents electrostatic preconcentration. Using positively charged micelles as ligand carriers overcomes this problem.

FGF-2 solutions (25 nM) containing increasing concentrations of dendrimers or sugars (from 0.5 nM to 500 μM) were flowed over the gold chip and the SPR sensorgrams were recorded (Figure 4). Positive (5 kDa heparin) and negative (**5**) controls were also measured (Supplemental Data). Binding of FGF-2 to immobilized hexasaccharide **6** was not significantly inhibited by **4**, or by the anti-ulcer drug sucrose octasulfate, which has been shown to bind to FGF [40] (Supplemental Data). As little as 500 nM **1** decreased the SPR response to 37%, whereas 500 μM monovalent **6** was required to produce a similar decrease of the signal (Figure 4). These results demonstrate the importance of multivalent presentation of heparin epitopes for enhancing protein binding, by mimicking the naturally occurring heparin-like oligosaccharides found on cell-surface proteoglycans.

FGF-2 Activity Assay

We determined the capacity of dendrimer **1** to influence the effect of FGF-2 on the activation of the mitogen-activated kinase signaling pathway. Phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) in response to FGF was monitored for this purpose. Fibroblast growth factor receptor 1-IIIc (FGFR1-IIIc)-transfected L6 rat skeletal muscle myoblasts [41] were starved for 24 hr without supplements in the medium until they reached quiescence and only weak bands of phosphorylated ERK1/2 (P-ERK1/2) were detected by western blot analysis. Then, the cells were treated with medium without FGF-2 or stimulated with FGF-2 (5 ng/ml) for 10 min (Figure 5). Different concentrations of dendrimer **1**, PAMAM dendrimer **5**, or hexasaccharide **6** (ranging from 50 nM to 5 μM) were incubated for 1 hr at room temperature with FGF-2 (5 ng/ml), and the cells were then incubated for

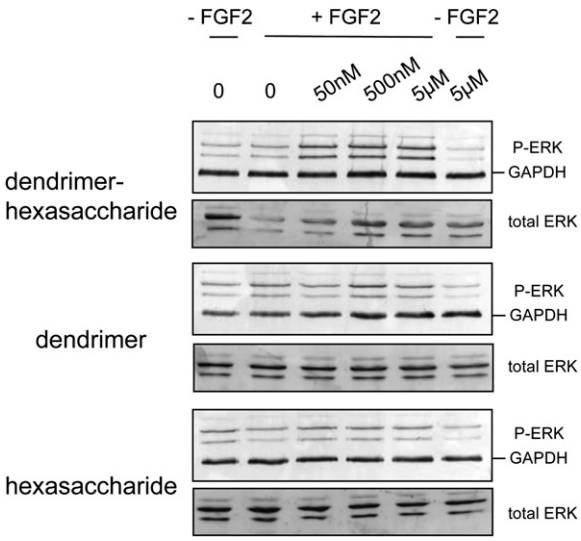

Figure 5. FGF-2 Activity Assay

L6 rat skeletal muscle myoblasts stably transfected with an FGFR1-IIIc expression vector were rendered quiescent and subsequently treated with medium without FGF-2 (−FGF2) or with FGF-2 (+FGF2) (5 ng/ml) for 10 min. Different concentrations of **1**, **5**, or **6** were incubated for 1 hr at room temperature with FGF-2, and cells were then incubated for 10 min with these mixtures. Treated and untreated cells were analyzed by western blotting with antibodies against phospho-ERK1/2, total ERK1/2, and GAPDH. The concentrations of **1**, **5**, or **6** are indicated at the top of each lane. Concentration of **1** is moles of heparin oligosaccharide.

10 min with these mixtures. Similar results were obtained without preincubation (data not shown). The cells were harvested and lysed, and the lysates were analyzed by western blotting with antibodies against P-ERK1/2, total ERK1/2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Dendrimer **1** without FGF-2 was not able to activate ERK1/2 phosphorylation. The low concentration of FGF-2 we used caused only a minor increase in ERK1/2 phosphorylation under our experimental conditions (Figure 5 and data not shown). However, when cells were treated with a mixture containing FGF-2 and dendrimer **1**, a significant increase in the P-ERK1/2 signal was detected compared to FGF-2 alone (Figure 5). As little as 50 nM **1** was sufficient to enhance activation, suggesting that **1** is a strong stimulator of FGF-2 signaling. Nonfunctionalized dendrimer **5** and hexasaccharide **6** did not affect ERK1/2 phosphorylation in the presence or absence of FGF-2. Heparin (15 U/ml) strongly potentiated the effect of FGF-2 under the same conditions (data not shown).

FGF signaling involves binding to specific cell-surface tyrosine kinase receptors (FGFRs) and is tightly regulated by heparin-like glycosaminoglycans (HLGAGs) that facilitate the formation of FGF-FGFR complexes [42–44]. The various FGFs require HLGAGs for biological activity, presumably to generate and stabilize ternary signaling complexes with FGFRs. High-resolution X-ray crystal structures of FGF-FGFR-heparin complexes [45–47] have provided insights into structural aspects of these interactions and demonstrated the complexity of the molecular mechanism involved in heparin-mediated FGF signaling.

The results of this kinase assay suggest that **1**, displaying multiple copies of a hexasaccharide containing the trisulfated repeating unit of the major region of heparin, is able to promote the formation of FGF-FGFR complexes and activate FGF-2-mediated signaling. In addition, comparison of ERK1/2 phosphorylation by FGF-2 with or without **1** indicates that the hexasaccharide-coated dendrimer generates the ternary complex more efficiently than heparan-sulfate chains on the surface of myoblasts. The stimulation of kinase production by **1** is similar to that with soluble heparin. Interestingly, hexasaccharide **6** did not stimulate ERK1/2 phosphorylation. This finding emphasizes the importance of multivalent display to activate FGF-2 signaling and is in agreement with reports showing that longer heparin sequences are required for FGF activation and mitogenesis [13, 14, 48, 49].

SIGNIFICANCE

Synthetic, amine-functionalized heparin oligosaccharides can be attached to hyperbranched macromolecules, such as dendrimers, to create novel heparin conjugates for applications in glycobiology. We demonstrate that the multivalent presentation of heparin epitopes on the surface of PAMAM dendrimers amplifies the binding affinity of heparin oligosaccharides to heparin-binding proteins such as FGF-2 drastically. Carbohydrate microarray and SPR experiments require only miniscule amounts of dendrimer and protein to determine binding affinities. Well-defined heparin dendrimers function as heparin mimetics to target heparin-mediated biological processes. To highlight this potential, we demonstrate that FGF-2 mitogenic signaling in L6 myoblasts can be activated by heparin dendrimers. These synthetic analogs may avoid the side effects associated with natural heparin.

FGFs are potent regulators of cell proliferation and survival, and a recombinant form of human keratinocyte growth factor (FGF-7) is already on the market for the treatment of severe oral mucositis [50]. Heparin dendrimers may provide an opportunity to discover novel therapeutic agents for tissue repair, regeneration, and cytoprotection [51, 52] by modulating heparin-FGF interactions. Blocking interactions between heparan sulfate and different protein families, such as selectins and chemokines that play a key role in inflammatory processes, may be another area of dendrimer application. New anti-inflammatory drugs with minimal side effects may be based on synthetic heparin dendrimers.

EXPERIMENTAL PROCEDURES

General Methods

All chemicals used were reagent grade and used as supplied. All aqueous solutions were made from nanopure water. Starburst PAMAM

dendrimer generation 2.5 containing 32 sodium carboxylate surface groups was purchased from Sigma-Aldrich (St. Louis, MO, USA). ^{1}H-NMR spectra were recorded on a Varian VXR-300 (300 MHz) or Bruker ARX300 (300 MHz) spectrometer. Microcon centrifugal filter units were purchased from Millipore (Billerica, MA, USA). Solutions used for chip hybridizations were sterile filtered through a 0.2 μm syringe filter prior to use. Deaminated heparin (5 kDa) was purchased from Sigma-Aldrich. Recombinant human basic fibroblast growth factor (FGF-2) and rabbit polyclonal anti-human FGF-2 were purchased from Pepro-Tech EC (London, UK). Goat anti-rabbit IgG labeled with Alexa Fluor 546 dye was purchased from Invitrogen (Carlsbad, CA, USA). Code-Link slides were purchased from Amersham Biosciences. Microarrays were constructed using a sciFLEXARRAYER noncontact printer from Scienion AG (Berlin, Germany). HybriSlip hybridization covers were purchased from Grace BioLabs (Bend, OR, USA). Slides were scanned using an LS400 scanner from Tecan (Männedorf, Switzerland) and quantified using Gene Spotter (MicroDiscovery GmbH, Berlin, Germany) software. SPR measurements were performed on a BIAcore 3000 (BIAcore, Uppsala, Sweden) operated by BIAcore control software. HBS-EP buffer (10 mM HEPES [pH 7.4], 150 mM NaCl, 3 mM EDTA, 0.005% v/v surfactant P20) and CM5 chips were purchased from BIAcore.

Preparation of Glycodendrimers 1–4

Activation of PAMAM Dendrimer Carboxylate Groups

Commercially available methanolic PAMAM solution was concentrated and coevaporated with CH_2Cl_2 twice under reduce pressure. The residue (20 mg, 3.2 μmol) was dissolved in anhydrous DMSO (2 ml). EDC (23.6 mg, 122 μmol, 1.2 equivalents per carboxylate group) and NHS (14.2 mg, 122 μmol, 1.2 equivalents per carboxylate group) were added and the reaction mixture was stirred under an argon atmosphere for 24 hr.

Dendrimer 4. Et_3N (10 μl) and monosaccharide **9** (3.0 mg, 5.6 μmol, 1.1 equivalents per carboxylate group) were added to an aliquot of the activated PAMAM solution (100 μl, 0.16 μmol). More DMSO (100 μl) was then added to aid in solubility of the sugar. The reaction mixture was stirred under argon for 24 hr and then lyophilized to remove the DMSO. The residue was dissolved in water and purified by centrifugal ultrafiltration (Microcon 3 kDa, 40 min, 14,000 rpm), washing twice with water. Lyophilization in water afforded the corresponding dendrimer **4** as a white powder (1.4 mg). Some batches were submitted to an additional purification step through Sephadex G-25 (prepacked PD-10 column; Amersham Biosciences) in order to remove the glycerin which coats Microcon ultrafiltration membranes and eventually contaminates the dendrimer sample. The ratio of sugar to dendrimer was 8.6 (27% loading of monosaccharide **9** on the dendrimer) based on the ^{1}H-NMR spectrum in D_2O (300 MHz) (Supplemental Data). The molecular weight of **4** (10.5 kDa) was estimated based on NMR integration. To estimate the molecular weights, it was assumed that all the sulfate groups of the sugar and the carboxylic acid groups of the dendrimer were present as sodium salts.

Dendrimer 3. Et_3N (10 μl) and monosaccharide **8** (3.2 mg, 5.6 μmol, 1.1 equivalents per carboxylate group) were added to an aliquot of the activated PAMAM solution (100 μl, 0.16 μmol). More DMSO (100 μl) was then added to aid in solubility of the sugar. The reaction mixture was stirred under argon for 24 hr and then lyophilized to remove the DMSO. The residue was dissolved in water and purified by centrifugal ultrafiltration (Microcon 3 kDa, 40 min, 14,000 rpm), washing twice with water. Lyophilization in water afforded the corresponding dendrimer **3** as a white powder (1.6 mg). The ratio of sugar to dendrimer was 7.6 (24% loading of monosaccharide **8** on the dendrimer) based on the ^{1}H-NMR spectrum in D_2O (300 MHz) (Supplemental Data). The molecular weight of **3** (10.3 kDa) was estimated based on NMR integration.

Dendrimer 2. Et_3N (10 μl) and disaccharide **7** (4.7 mg, 5.6 μmol, 1.1 equivalents per carboxylate group) were added to an aliquot of the activated PAMAM solution (100 μl, 0.16 μmol). More DMSO (100 μl) was then added to aid in solubility of the sugar. The reaction mixture was stirred under argon for 24 hr and then lyophilized to remove the DMSO. The residue was dissolved in water and purified by centrifugal ultrafiltration (Microcon 3 kDa, 40 min, 14,000 rpm), washing twice with water. Lyophilization in water afforded the corresponding dendrimer **2** as a white powder (2 mg). The ratio of sugar to dendrimer was 7.3 (23% loading of disaccharide **7** on the dendrimer) based on the ^{1}H-NMR spectrum in D_2O (300 MHz) (Supplemental Data). The molecular weight of **2** (12.1 kDa) was estimated based on NMR integration.

Dendrimer 1. Et_3N (10 μl) and hexasaccharide **6** (6.1 mg, 2.8 μmol, 1.1 equivalents per carboxylate group) were added to an aliquot of the activated PAMAM solution (50 μl, 0.08 μmol). More DMSO (100 μl) was then added to aid in solubility of the sugar. The reaction mixture was stirred under argon for 24 hr and then lyophilized to remove the DMSO. The residue was dissolved in water and purified by centrifugal ultrafiltration (Microcon 10 kDa, 30 min, 14,000 rpm), washing twice with water. Lyophilization in water afforded the corresponding dendrimer **1** as a white powder (1.8 mg). The ratio of sugar to dendrimer was 8.7 (27% loading of hexasaccharide **6** on the dendrimer) based on the ^{1}H-NMR spectrum in D_2O (300 MHz) (Supplemental Data). The molecular weight of **1** (24.9 kDa) was estimated based on NMR integration.

Carbohydrate Microarray Experiments

Preparation of Amine-Functionalized 5 kDa Heparin

Deaminated 5 kDa heparin (2 mg, 0.4 μmol) was dissolved in MES buffer (0.4 ml, 50 mM [pH 6.8]). 1,11-diamino-3,6,9-trioxaundecane [53] (0.8 mg, 4 μmol) in DMF (25 μl) and $NaCNBH_3$ (50 μl of a 0.16 M solution in water) were added and the reaction mixture was shaken at room temperature for 18 hr. To remove linker excess, the solution was submitted to centrifugal ultrafiltration (Microcon 3 kDa, 40 min, 14,000 rpm), washing three times with water. The residue was diluted with water and lyophilized to give a white solid (2 mg) that was stored at −20°C until use.

Heparin Array Fabrication

Amine-functionalized heparin (average molecular weight 5 kDa) was dissolved in sodium phosphate buffer (50 mM [pH 9.0]) and spatially arrayed onto *N*-hydroxysuccinimide (NHS)-activated CodeLink slides by use of an automated arraying robot. Each glass slide was subdivided into eight different sections and heparin was printed in each section, at two concentrations (0.5 and 1 mM) in 17 replicates, in order to examine eight different conditions on each slide using an eight-well hybridization chamber. Slides were printed in 50% relative humidity at 22°C, followed by incubation overnight in a saturated NaCl chamber that provides a 75% relative humidity environment. The robot delivered 1 nl (approximately) of heparin solution and the resulting spots had an average diameter of 200 μm with a distance of 500 μm between the centers of adjacent spots. Slides were then washed three times with water to remove the unbound carbohydrates from the surface. Remaining succinimidyl groups were quenched by placing slides in a solution preheated to 50°C that contained 100 mM ethanolamine in sodium phosphate buffer (50 mM [pH 9.0]) for 1 hr. Slides were rinsed several times with distilled water, dried by centrifugation, and stored in a dessicator prior to use.

Binding Assay

As described above, heparin was spotted in eight predetermined places on the glass slide. After attaching an eight-well hybridization chamber to the slide, each block was incubated with 40 μl of a mixture of FGF-2 (29 nM) and a competitor in PBS buffer (10 mM [pH 7.5]) containing BSA (1%) for 1 hr at room temperature. Glycodendrimers **1**–**4**, PAMAM dendrimer **5**, oligosaccharides **6**–**9**, sucrose octasulfate, and deaminated 5 kDa heparin were added as competitors at concentrations ranging from 0.5 nM to 2.5 mM (Figure 3; Supplemental Data). A solution of FGF-2 (29 nM) without competitor was used as positive control. The arrays were washed twice with PBS (10 mM [pH 7.5]) containing 1% Tween 20 and 0.1% BSA, twice with water, and then centrifuged for 3 min to ensure dryness. For detection of bound FGF-2, arrays were incubated with polyclonal rabbit anti-human FGF-2 (20 μg/ml) and then washed as above. The array incubation was performed as follows: 100 μl of antibody solution was placed

between the array slide and a plain coverslip and incubated for 1 hr at room temperature. Finally, Alexa Fluor 546-labeled anti-rabbit IgG (20 μg/ml) was used to detect bound rabbit primary antibodies, and washed as above. All the competition assays were carried out at least in duplicate.

Image Acquisition and Signal Processing

Heparin arrays were scanned by using an LS400 scanner, and fluorescence intensities from these scans were integrated on Gene Spotter software. Signal to background was typically ≥50:1. The local background was subtracted from the hybridization signal of each separate spot and the mean intensity of each spot was used for data analysis. Spot finding was automatically performed, followed by manual fitting to correct spot deviations. Data presented are the average of 17 spots on the same array at 1 mM; errors are the standard deviations for each measurement. Similar results were obtained by measuring heparin microspots at 0.5 mM (data not shown).

Data Analysis

Curve fitting and calculation of IC_{50} values were performed with the GraphPad Prism program (GraphPad Software, San Diego, CA, USA). The fluorescence intensity was represented as a function of the logarithm of concentration of competitor. The data were fitted to a sigmoidal dose-response curve with a constant slope: Y = Bottom + [(Top-Bottom)/(1 + $10^{(\log IC_{50} - X)}$)], where X is the logarithm of competitor concentration and Y represents the fluorescence intensity (response) that starts at Top and goes to Bottom with a sigmoid shape. For the dendrimers, the concentration is moles of oligosaccharide.

SPR Measurements

Immobilization of Hexasaccharide 6 on a CM5 Sensor Chip

Hexasaccharide **6** was covalently bound to the sensor surface via the primary amino group using the following protocol: HBS-EP was employed as running buffer. The carboxymethylated dextran matrix (CM5 chip) was first activated at a flow rate of 5 μl/min by using a 15 min (75 μl) injection pulse of an aqueous solution containing *N*-hydroxysuccinimide (NHS, 0.05 M) and *N*-ethyl-*N*′-(dimethylaminopropyl) carbodiimide (EDC, 0.2 M). Next, a 50 μl injection of **6** (500 μg/ml) in 5 mM sodium phosphate buffer (pH 7.4) containing NaCl (1 M) was flowed over the activated surface followed by an additional 50 μl injection of **6** (500 μg/ml) in 5 mM sodium phosphate buffer (pH 7.4) containing hexadecyltrimethylammonium chloride (1 mM). Remaining activated sites on the chip surface were blocked with a 35 μl injection of an ethanolamine hydrochloride solution (1 M [pH 8.5]). A second flow cell of the CM5 chip was used as negative control after activation with EDC and NHS followed by treatment with ethanolamine solution as described above. An increase of approximately 300 response units was detected in the flow cell containing **6** when compared to the control cell.

Measurement of FGF-2-Hexasaccharide 6 Interactions in the Presence of Soluble Competitors

A 30 μl injection of a series of mixtures containing FGF-2 (25 nM) and competitor (0.5 nM to 500 μM) in HEPES buffer (10 mM HEPES [pH 7.4], 300 mM NaCl, 3 mM EDTA, 0.01% v/v Tween 20) was made at a flow rate of 10 μl/min. The concentration of NaCl in the HEPES buffer was increased to 300 mM to eliminate nonspecific background binding [34]. At the end of the sample injection, the same buffer was flowed over the sensor surface for 30 s, and then the chip surface was regenerated for the next sample by injecting a 50 μl pulse of 4 M NaCl followed by a 50 μl pulse of 0.5% sodium dodecyl sulfate (SDS), both at 50 μl/min. The response was monitored as a function of time (sensorgram). All experiments were carried out at least in duplicate.

ERK1/2 Activation Assay

Rat skeletal muscle myoblasts stably expressing the IIIc variant of FGFR1 [41] were grown to confluency in Dulbecco's modified Eagle's medium (Sigma, Munich, Germany), supplemented with 10% fetal calf serum (BioConcept Amimed, Allschwil, Switzerland) and 1% penicillin/streptomycin, serum starved for 24 hr, and subsequently treated with different combinations of FGF-2 (5 ng/ml; Roche, Rotkreuz, Switzerland) and additives (see below) for 10 min at 37°C. FGF-2 was incubated for 1 hr at room temperature in the presence or absence of different concentrations of heparin (15 U/ml; Sigma), **1**, **5**, or **6** and subsequently added to the starved cells. After treatment, cells were lysed on ice with 200 μl of lysis buffer (20% glycerin, 100 mM Tris/HCl [pH 8], 3 g/l SDS, 5% β-mercaptoethanol, 2 mM EDTA, 0.5 mM AEBSF, 1 mM $Na_2P_2O_7$, 1 mM Na_3VO_4) per 6 cm plate. Equal amounts of total protein were analyzed by western blotting using antibodies against phosphorylated ERK1/2 (P-ERK1/2; Cell Signaling, Danvers, MA, USA), total ERK1/2 (Cell Signaling), or glyceraldehyde 3-phosphate dehydrogenase (GAPDH; HyTest Ltd., Turku, Finland). Detection of antibody-binding proteins was performed with the alkaline phosphatase detection system (Promega, Madison, WI, USA).

Supplemental Data

Supplemental Data include ^{1}H-NMR spectra of glycodendrimers, additional pictures of eight-well arrays, IC_{50} determination for 5 kDa heparin, and negative and positive control experiments for the microarray and SPR assays and are available at http://www.chembiol.com/cgi/content/full/14/8/879/DC1/.

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