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Effect of Heparin Oligomer Chain Length on the Activation of Valvular Interstitial Cells

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Abstract

A key event in connective tissue remodeling involves the transformation of fibroblasts to myofibroblasts, also revealed by expression of α -smooth muscle actin (α -SMA). However, misregulation of this transition can lead to fibrosis, an overgrowth and hardening of tissue due to excess extracellular matrix deposition, a process that is linked to heart valve disease and many others. Both disease treatment and regenerative strategies would benefit from strategies for the controlled delivery and presentation of bioactive factors that can promote or suppress this transformation. In this regard, the ability of heparin to complex a plethora of growth factors offers a broad range of possibilities for this purpose. Here, the effects of heparin chain length and structure on valvular interstitial cell (VIC) phenotypic expression were explored. Heparin from porcine intestinal mucosa was depolymerized with heparinase and fractionated to obtain oligosaccharides of different sizes. VICs cultured with octasaccharides and decasaccharides exhibited higher expression of α -SMA when compared to other saccharides and full-length heparin. No activation of VICs was observed in response to full-length heparin presence in media.

Keywords

Heparin; valvular interstitial cells; myofibroblasts

Introduction

Fibrosis develops when inherent wound-healing processes are somehow impaired. In healthy tissue, fibroblasts deposit collagen to form a provisional matrix that enables injuries to be repaired. However, when the organ is diseased, the excessive production of matrix proteins results in pathological scarring, or fibrosis. In the case of the aortic heart valve, the overproduction of extracellular matrix leads to stiffness and inflexibility of the leaflet, which corresponds to malfunction and possible heart failure. While it is known that valvular interstitial cells (VICs) are the main cell population in valve tissue and activation of this fibroblast-like cell is responsible for cardiac fibrosis, the origin and controlling factors behind the fibroblast-to-myofibroblast transition remain unclear. Under certain stimuli, VICs activate to the myofibroblast phenotype, as characterized by an over-secretion of collagen and the expression

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of the contractile protein alpha-smooth muscle actin (α -SMA). However, this phenotype plasticity of VICs is regulated by both physical and chemical cues in their microenvironment.

Transforming growth factor beta (TGF- β)¹ and connective tissue growth factor (CCN2/CTGF) have been identified as stimulators of myofibroblast activation.²⁻⁴ More recently, basic fibroblast growth factor (bFGF) was found to antagonize TGF- β -mediated induction of α -SMA expression.⁵ bFGF binds to cells through two types of receptors that require the presence of heparin to function.⁶ FGF receptor (FGFR) dimerization is induced by oligomerization of FGF molecules, and both its activation and FGF-stimulated biological responses require heparin-like molecules.⁷⁻⁹

Glycosaminoglycans (GAGs) such as heparin are linear, sulfated polysaccharides containing between 20 and 1500 alternating units of L-iduronic and D-glucosamino disaccharides and are located primarily on the surface of cells and in the extracellular matrix.¹⁰ At physiological pH, heparin has a high anionic charge from deprotonated sulfate groups, resulting in high water retention and the ability to interact with positively charged molecules, such as basic proteins, through non-covalent electrostatic forces. Several studies have focused on the role of GAGs in the FGF-FGFR complex, and examined the effect of heparin size and chemical structure required to form active signaling complexes.¹¹⁻¹⁵ Similar results have been reported for many other heparin-binding proteins: The minimal sequence of heparin required for binding with TFPI (Tissue Factor Pathway Inhibitor) consists of 12-14 saccharides, and 2-O-sulfate, 6-O-sulfate and N-sulfate groups.¹⁶ For the enhancement of inhibitory activity, 6-O-sulfate groups represent the main interactions compared to 2-O-sulfate groups.¹⁷ Heparin chains longer than 22 saccharides and with sulfation on the 6-O and N positions of glucosamine units enhance VEGF binding to fibronectin.¹⁸ Moreover, some preclinical data shows how heparin octa- and decasaccharides play a role in reducing microvessel density.¹⁹

The aim of this contribution was to study how the myofibroblast phenotype in VICs might be regulated by a specific heparin sequence. Since it has already been shown that the presence of heparin in a VIC's microenvironment can promote α -SMA expression and increased collagen production,^{20,21} we sought to elucidate which moieties in the heparin chemical structure might be involved in this process. Particularly, in heparin samples that appear to be very heterogeneous and polydisperse, undesired or unexpected cell responses may result. Consequently, specific sequences might be used to sequester and/or deliver bFGF and trigger VIC (de)activation.

Materials and Methods

Materials

Heparin (from porcine intestinal mucosal) sodium salt, heparin lyase I (heparinase, E.C. 4.2.2.7) and mouse monoclonal Anti- β -Tubulin I (clone SAP.4G5) were purchased from Sigma, Saint Louis, MO. Mouse monoclonal Anti- α Smooth Muscle Actin, clone 1A4, was purchased from Abcam Inc., Cambridge, MA and used at 1:75 dilution. Alexa Fluor® 488 F (ab')₂ goat anti-mouse IgG was purchased from Molecular Probes, Eugene, OR. Bio Gel P-2 was obtained from BioRad, Richmond, CA. HPLC was performed on a 5 μ m C₁₈ Waters Nova Pak column from Agilent, Bellefonte, PA of dimensions 3.9×150 mm.

Preparation of heparin oligosaccharides

The heparin oligosaccharide mixture was prepared by controlled enzymatic depolymerisation of heparin sodium salt with heparin lyase I as previously described.²² Briefly, to a solution of 500 mg of heparin in 5 ml of sterilized digestion buffer (5 mM TRIS, 50 mM NaCl, 2 mM CaCl₂, 0.1 mg/ml bovine serum albumin, pH 7.5), 40 mIU of heparin lyase I was added at a

concentration of 0.24 mIU enzyme/mg of heparin. The solution was incubated at 30 °C until the digestion was complete (72 h). The oligosaccharide fraction from disaccharides through tetradecasaccharides was desalted by a Bio-Gel P-2 column and freeze-dried. The progression of the enzymatic reaction was monitored by the increase in the UV absorbance at 232 nm.

Separation of the oligosaccharide mixture was performed by reversed-phase ion-pairing chromatography (RP-HPLC) as described elsewhere.²³ A semipreparative octadecyl (C₁₈) silica column and a UV (232 nm) detector was employed. The mobile phases were acetonitrile-water containing tributylamine and ammonium acetate. A linear gradient elution from 20% to 65% acetonitrile over 120 min at a flow-rate of 1 ml/min was used. The oligosaccharide fractions were freeze-dried to remove the volatile ion-pairing reagent and desalted.

Cell culture and seeding

VICs were isolated from porcine aortic valves²⁴ and cultured in growth media consisting of 15% FBS, 2% penicillin/streptomycin and 0.2% gentamicin in Media 199 (Invitrogen Corp.) at 37 °C in a 5% CO₂ environment. Seeding was performed in tissue-culture treated 96-well flat bottom polystyrene plates at 10,000 cells/cm² (Falcon 3072, BD Biosciences, Bedford, MA).

Cell-based FLISA (Fluorophore-linked Immunosorbent Assay)

Expression of α -SMA was quantified and normalized to β -1-Tubulin expression using an assay similar to the cell-based ELISA.²⁵ After cells were fixed and permeabilized, the primary antibody was applied in 1% BSA in PBS and subsequently, a fluorescently labeled secondary antibody. α -SMA was calculated by fluorescence analysis at 535 nm (Wallac Victor2 Multilabel Counter, PerkinElmer). All cell data is shown as the mean of 3 replicates, and each experiment was repeated 3 times.

Results and Discussion

Heparinase I cleaves heparin at the linkage between N-sulfo- α -D-glucosamine (a 6-O-sulfo group can be present) and 2-O-sulfo- α -L-iduronic acid by an elimination mechanism, originating in an unsaturated 2-O-sulfo uronic acid at the non-reducing end of the oligosaccharide product (Fig. 1a).²⁶ Thus, the oligosaccharides formed by the heparinase I-catalyzed depolymerization of heparin differ in size by a disaccharide repeat units.

A semi-preparative HPLC octadecyl (C₁₈) silica column, a volatile ion-pairing reagent (tributylammonium acetate), and a gradient of ammonium acetate were used to separate the oligosaccharides ranged from disaccharides (dp2) to tetradecasaccharides (dp14). The resulting chromatogram is displayed in Fig. 1b. Native heparin and heparin oligosaccharides were added for 3 days to different VIC cultures at a concentration of 100 μ g/ml in order to assay the influence in myofibroblast phenotype. Fig. 2 represents the effect of different heparin sequences on VIC α -SMA production and shows the percent change versus the untreated culture, used as control. The presence of tetrasaccharides and longer fragments increased protein expression with increasing fragment length, but only octa- and decamers had a significant effect in α -SMA production. Dodecamers and full-length heparin had no effects on VIC phenotype.

A considerable number of experiments show that only heparin fragments long enough to extend over the FGF and FGFR are able to promote growth factor activity. Therefore, sequences too short would bind the FGF, blocking the interaction with full-size heparin, and inhibiting its activity.²⁷⁻²⁹ For instance, in human colon carcinoma, heparin dp12 activates FGF-2, whereas heparin dp8 and dp10 inhibit the biological activity.³⁰ Also, in the morphological

transformation of astrocytes, heparin dp8 and dp10 were inhibitors of the process, while native heparin and dp14 promoted astrocytic stellation, however the dp4 had no effects.³¹ According to these findings, heparin samples with high concentrations of deca- or octa-saccharides could easily block growth factor pathways and therefore increase α -SMA in VICs, while species containing 12 or more saccharides would support biological activity.

Native commercial heparin from the same source revealed contradictory effects in the phenotypic expression of VICs. Therefore, two different batches, numbers 035 and 114, were analyzed in this regard. The starting heparins were examined by GPC (Fig. 3), showing a different molecular weight distribution between commercial heparin batches as summarized in Table 1. Batch number 114 has been reported to induce actin expression in VICs, while we have found that batch 035 does not have any effect in protein production. Batch 114 has higher molecular weight and polydispersity, but smaller degree of short heparin chains than batch 035. This molecular weight distribution pattern may explain the biological behavior as stated previously.

The extent of heparin N-acetylation is also important in the inhibitory activity induced by certain heparin saccharides.³² Heparin batch 035 has a lower degree of free amino groups (2%) than heparin batch 114 (7%), which means a higher N-sulfation and/or acetylation pattern. This observation reinforces the marked differences in biological activity between the two samples and might be also related to the importance of sulfate groups on the activation of cell signaling. Following this reasoning, charge distribution might also be considered important in biological activity of heparin since electrostatic interactions play an important role in glycosaminoglycan-growth factors contacts. Heparin batch 114 contains less negatively-charged groups per saccharide than heparin batch 035 and has a higher effect in VICs phenotype. This induction of α -SMA expression is consistent with the biological activity of dp10, which generally has a lower charge than other oligosaccharides such as dp12.³³

The present results can be used as alternative data to control valvular interstitial cell activation to myofibroblast, characterized by the expression of α -SMA, and the future development of culture environments for regenerative medicine applications. It is generally accepted that FGFs are involved in these processes and require heparin oligosaccharides larger than dp6 for activation, whereas tetrasaccharides are required for efficient binding activity. However, some recent studies reveal that heparin decasaccharide and the 6-desulfated heparin decasaccharide inhibit binding of the FGF-FGFR complex to heparin.^{34,35} Ferning et al.³⁶ also showed discrepancies between the binding parameters of FGF2-heparin and the stimulation of cell proliferation. Octa- and decasaccharides had the highest association constant, however, were less potent in proliferation assays than dp4 sequences. There is also evidence in the literature that the activity of particular heparin-binding factors can be inhibited by distinctive oligosaccharides (*i.e.*, dp8) that can bind the factors (*i.e.*, FGF2) but cannot form functional signaling complexes.^{37,38} In the same line, Gallagher et al.³⁹ described how heparin dp6 is the shortest FGF-binding saccharide, dp8 the minimum needed for FGF2 dimerization, and a complex formation is observed for dp12 oligosaccharides. Nevertheless, data also suggest that some heparin species from dp6 to dp12 form unproductive combinations of FGF2-FGFR leading to mitogenesis inhibition, indicating that binding of FGF2 with heparin oligosaccharide is not enough to establish transmembrane signaling. Therefore, the observation that dp8 and dp10 sequences induce α -SMA through the inhibition of FGF-FGFR productive formation, concur with previous findings. The heterogeneity in heparin samples stems from the number of disaccharide repeat units present, as well as the numerous sites susceptible to chemical modification.⁴⁰ This wide variability in heparin chain sequences emphasizes the importance of the source, the isolation methodology and the procedure to obtain the saccharides in heparin's capacity to inhibit FGFs activity. Overall, our data suggest that the activity of particular heparin-binding factors can be inhibited by distinctive oligosaccharides that can bind targeted

factors but cannot form functional signaling complexes. Results are consistent with literature and point to alternative directions in the identification of heparin sequences to control valvular interstitial cell de/activation for tissue engineering applications⁴¹ as well as other incipient uses such as inhibition of virus infection or cancer that can be applied in devices for pharmacology or medicine.⁴²⁻⁴⁴

Conclusion

In conclusion, our data supports the hypothesis that smaller saccharides, such as di- to hexasaccharides can interact with bFGF to promote dimerization and activate FGFR, as well as saccharides longer than dodecasaccharides, that are large enough to act as a bridge between bFGF and FGFR. Octa- and decasaccharides, on the other hand, are not long enough to connect two FGF molecules. Therefore, they might be able to bind FGF without causing dimerization or activation of the FGFR.

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Figure 1. Structure of a representative heparin-derived oligosaccharide produced by heparinase I-catalyzed depolymerization of heparin (a) and HPLC profile of heparin-derived oligosaccharides (b).

Figure 2.

Modification of culture media with different chain length heparin fragments varies expression of α -SMA of VICs. Protein expression is normalized to β -Tubulin, and presented as % of expression on media in polystyrene. VICs were cultured for 72 h before assay. Data is presented as mean $\pm$ standard deviation. At minimum, three samples were represented for each data point. Data were compared using a two tailed, unpaired *t*-test, and *p* values less than 0.05 were considered statistically significant. (* indicates *p* < 0.05).

Figure 3.
Heparin molecular weight distributions from different batches (114 and 035) as analyzed by GPC. Molar mass values are based on poly(methacrylic acid) standards.

Table 1
Molar mass distribution (Da) of full length heparin from different batches as determined by GPC.

Heparin batch	M _n	M _w	MP	M _z	M _{z+1}	PDI
035	5139	7248	9380	8713	9749	1.41
114	7952	11787	16629	14697	16783	1.48