Structural determinants of antiproliferative activity of heparin on pulmonary artery smooth muscle cells

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Garg, Hari G., B. Taylor Thompson, and Charles A. Hales. Structural determinants of antiproliferative activity of heparin on pulmonary artery smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 279: L779–L789, 2000.—In addition to its anticoagulant properties, heparin (HP), a complex polysaccharide covalently linked to a protein core, inhibits proliferation of several cell types including pulmonary artery smooth muscle cells (PASMCs). Commercial lots of HP exhibit varying degrees of antiproliferative activity on PASMCs that may due to structural differences in the lots. Fractionation of a potent antiproliferative HP preparation into high and low molecular weight components does not alter the antiproliferative effect on PASMCs, suggesting that the size of HP is not the major determinant of this biological activity. The protein core of HP obtained by cleaving the carbohydrate-protein linkage has no growth inhibition on PASMCs, demonstrating that the antiproliferative activity resides in the glycosaminoglycan component. Basic sugar residues of glucosamine can be replaced with another basic sugar, i.e., galactosamine, without affecting growth inhibition of PASMCs. N-sulfonate groups on these sugar residues of HP are not essential for growth inhibition. However, O-sulfonate groups on both sugar residues are essential for the antiproliferative activity on PASMCs. In whole HP, in contrast to an earlier finding based on a synthetic pentasaccharide of HP, 3-O-sulfonation is not critical for the antiproliferative activity against PASMCs. The amounts and distribution of sulfonate groups on both sugar residues of the glycosaminoglycan chain are the major determinant of antiproliferative activity.
antiproliferative activity on aortic vascular SMCs. Briefly, they suggested that 1) the anticoagulant and antiproliferative properties of HP reside in different HP domains, 2) 3-O-sulfate on the internal glucosamine residue of a chemically synthesized pentasaccharide (Fig. 1) (63) is critical for the growth-inhibitory capacity of the pentasaccharide (11), 3) the dodecasaccharide of HP contains the full antiproliferative activity (10), 4) 2-O-sulfonation of glucuronic acid in HP is not essential for antiproliferative activity (75), 5) the relationship between the degree of N-desulfonation and the inhibition of cell proliferation is not straightforward (71), 6) acetylation of the N positions of the N-desulfonated glucosamine residues does not seem to restore the antiproliferative activity (71), and 7) both O-sulfonation and N-sulfonation are important for antiproliferative activity (71).

Because vascular remodeling with SMC hypertrophy and hyperplasia contributes to the high pulmonary vascular resistance seen in primary as well as secondary pulmonary hypertension, interest continues in HP as a possible therapeutic agent to reverse vascular remodeling. In recent years, efforts have been made to establish which domain of the HP polysaccharide is related to the inhibition of growth of PASMCs. Several studies employing the following strategies, mild chemical modification, fractionation, or enzymatic degradation, have appeared. This review provides an update on the effects of HP and its fragments on the inhibition of growth of PASMCs.

HEPARIN STRUCTURE

Arrangement of sugars in glycosaminoglycan chains. HP is one of the members of the class of glycosaminoglycans (GAGs; Table 1) (26) and consists of alternating residues of a uronic acid (either β-D-glucuronic acid or α-L-iduronic acid) with a hexosamine (α-D-glucosamine) linked by 1→4-glycosidic linkages and covalently bound to serine residues of the core protein. It has various O-sulfonate, N-sulfonate, and N-acetyl substituents that are usually heterogeneously distributed along the GAG chains.

Protein core. The protein cores of HP are diverse and heterogeneous, vary in size from 20 to 150 kDa, and appear to share only the capacity to bear GAG chains. Repetitive serine-glycine sequences are found in the protein core of heparin. The GAG chains are connected to the protein core through a tetrasaccharide (D-GlcA-D-Gal-D-Gal-D-Xyl) known as the linkage region (Fig. 2) (26). HP helicity (i.e., secondary structural pattern or coiling of the GAG chains) in conjunction with chirality (i.e., configuration of the carbon atoms at the asymmetric centers present in HP) inherent in the constituent monosaccharides makes it a unique macromolecule. Low molecular weight (LMW) HPs currently used in the treatment of acute proximal deep vein thrombosis are obtained by cleaving the GAG chains from the protein core.

Differences in the structure of HP and heparan sulfate. HP and heparan sulfate (HS) originate from the same biosynthetic precursor, N-acetylated heparosan (Fig. 3). After the initial assembly of the N-acetylated heparosan polymer from monosaccharide precursors, biosynthesis proceeds much further for HP than for HS. The following steps are involved in the biosynthesis of heparin: 1) N-deacetylation followed by N-sulfation of the basic sugar glucosamine (GlcN), 2) glucuronate C-5 epimerization of the acidic sugar D-glucuronic acid (GlcA) to L-iduronic acid (IdoA), and 3) further O-sulfation of the

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Table 1. General composition of different glycosaminoglycans (A-B)ₙ

<table>
<thead>
<tr>
<th>Glycosaminoglycan</th>
<th>A</th>
<th>Linkage</th>
<th>B</th>
<th>Linkage</th>
<th>NAc</th>
<th>SO₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyaluronan</td>
<td>GlcA</td>
<td>β-(1→3)</td>
<td>GlcN</td>
<td>β-(1→4)</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Chondroitin sulfate</td>
<td>GlcA</td>
<td>β-(1→3)</td>
<td>GalN</td>
<td>β-(1→4)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dermatan sulfate</td>
<td>IdoA</td>
<td>α-(1→4)</td>
<td>GlcN</td>
<td>α-(1→4)</td>
<td>(+)</td>
<td>++</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>IdoA</td>
<td>α-(1→4)</td>
<td>GlcN</td>
<td>α-(1→4)</td>
<td>(+)</td>
<td>+++</td>
</tr>
<tr>
<td>Heparin</td>
<td>IdoA</td>
<td>α-(1→4)</td>
<td>GlcN</td>
<td>α-(1→4)</td>
<td>(+)</td>
<td>+++</td>
</tr>
</tbody>
</table>

(A-B)ₙ, repeating disaccharide units of glycosaminoglycans containing acidic (A) and basic (B) sugar residues; n, variable size; GlcA, glucuronic acid; IdoA, iduronic acid; GlcN, glucosamine; GalN, galactosamine; NAc, N-acetylated. Residues in parentheses, minor components. +, Partially N-acetylated glucosamine residues; ++ and ++++, presence of >1 sulfate group in repeating disaccharide units.
acidic and basic monosaccharides compared with those for HS (34, 37, 46, 47, 53).

These additional biosynthetic steps for HP result in a GAG with a content of sulfamino groups that exceeds its content of acetamido groups and the concentration of the O-sulfate groups exceeds that of N-sulfonate groups. Only such GAGs qualify to be called HP. All other polysaccharides are known as HSs (23). Twenty-four different disaccharide structures (IdoA/GlcA-GlcN) are possible from the combination of different sulfate residues in HP/HS GAG chains (Fig. 4).

**HP AND PULMONARY HYPERTENSION**

Thompson and Hales (66) have recently reviewed the effect of HP on pulmonary hypertension and the associated vascular remodeling. Briefly, in our laboratory, Hales et al. (29) have shown in a mouse model of chronic hypoxia that HP inhibited the medial smooth muscle increase in vessels associated with terminal bronchioles, reduced right ventricular systolic pressure, and partially prevented the increase in medial thickness of intra-acinar vessels after 26 days of hypoxia. HP did not alter the hematocrit and was effective at low doses that did not prolong the partial thromboplastin time. HP did not block the rise in right ventricular systolic pressure after acute hypoxia, indicating that HP prevented vascular remodeling through a mechanism that did not involve blockade of hypoxic vasoconstriction.

Subsequently, in a guinea pig model of chronic hypoxia pulmonary hypertension (67), Hassoun et al. (33) showed that certain commercial HP preparations given by continuous subcutaneous infusion resulted in a 50% reduction in medial thickness of alveolar duct vessels (Fig. 5) and completely prevented the medial smooth muscle increase in vessels associated with terminal bronchioles (Fig. 6). Moreover, our laboratory found that fully established hypoxic pulmonary hypertension in the guinea pig was substantially reversed by HP (32) and that HP by aerosol was effective (64). Our laboratory has also shown that HP lots even from the same company vary in their ability to inhibit SMC proliferation and hypertrophy (44) and that this variation correlates with the ability of these HPs to prevent hypoxic pulmonary hypertension (68). Rats were said to be resistant to HP, but Du et al. (22) found that a strongly antiproliferative HP was effective in rats.

**ANTIPROLIFERATIVE ACTIVITY OF HP AND ITS DERIVATIVES ON PASMCs**

Mechanisms contributing to HP inhibition of SMC growth. HP is a potent inhibitor of SMC proliferation (9, 28, 35, 68). Both anticoagulant and nonanticoagulant HP are reported to possess effective SMC antiproliferative activity (55). Although much attention has been focused on factors that stimulate SMC proliferation (62), very little is known about the mechanisms maintaining these cells in a quiescent state or about the reestablishment of a quiescent state after their proliferative response has been initiated.

Circulating HP binds to endothelial cells and is taken up by the reticuloendothelial system where it enters a cellular pool to be released at a later stage (38). Furthermore, HP binds to specific binding sites on SMCs and is internalized (14). Some antiproliferative effects are mediated by specific binding, although it is not clear whether internalization is essential. HP blocks the cell cycle at either the G0/G1 transition point (12) or at mid to late G1 progression (14, 49, 58) and may inhibit such cellular intermediate processes as protein kinase C activation, c-Fos and c-Myc induction (13, 77), activator protein-1/Fos-Jun binding activity, and posttransitional modification of Jun B (2, 7, 54). HP has also been shown to selectively block the protein kinase C pathway of Fig. 2. Structure of heparin (HP) demonstrating the peptide core with glycosaminoglycan chains attached through a linkage region tetrasaccharide: glucuronic acid (GlcA)-galactose (Gal)-Gal-xylose (Xyl).
mitogenic signaling (20) and the phosphorylation of mitogen-activated protein kinase (52).

We have demonstrated that PASMC mitogens such as platelet-derived growth factor and epidermal growth factor act through the Na\(^+\)/H\(^+\) antiporter by stimulating a one-for-one exchange of extracellular Na\(^+\) for intracellular H\(^+\) to cause intracellular alkalinization, a permissive first step for cell division (56). Furthermore, Dahlberg et al. (19) have demonstrated that antiproliferative HPs block Na\(^+\)/H\(^+\) exchange in a manner directly related to antiproliferative activity.

**Structure-function relationship.** To understand the structure-function relationship of the HP polysaccharide, we compared the antiproliferative activity of three commercially available HPs. These preparations were from Upjohn, Elkins-Sinn, and Choay Pharmaceuticals. The growth-inhibitory activity of these HPs...
on PASMCs varied and was in the order Upjohn > Elkins-Sinn > Choay (19). The properties of these HP preparations are summarized in Table 2.

3-O-sulfonation of glucosamine residue is not critical for antiproliferative activity. These three commercially available HPs were degraded with heparitinases I and II to evaluate the overall content of 3-O-sulfation of glucosamine in whole HPs to see whether the 3-O-sulfate content correlated with the antiproliferative effect. These enzymes were unable to degrade the components of HP containing 3-O-sulfate on the glucosamine residue into disaccharide units, and instead,
tetrasaccharides were formed (Fig. 7) (79). Thus the tetrasaccharide content in the digest correlates with the content of 3-O-sulfate. The oligosaccharide profiles of the three batches of HPs after digestion with heparitinases I and II demonstrated that the most potent HP (Upjohn) contained the least amount of tetrasaccharide, i.e., the least amount of 3-O-sulfate on glucosamine residues. These results suggest that the presence of 3-O-sulfonated glucosamine residues in whole HP are not an essential requirement for antiproliferative activity as previously reported (11) based on data derived from the synthetic pentasaccharide (63). The Δ-disaccharides [hexauronic acid (HUA)-glucosamine (GlcN)] liberated after heparitinase treatment of these three HPs were analyzed by us, and the results are given in Table 3 (25). The results show that the most potent Upjohn HP preparation had the largest amount of trisulfonated Δ-disaccharide.

Influence of molecular weight protein core and GAGs of HP on antiproliferative activity. Structure-function studies carried out by preparing discrete sizes of antiproliferative HP fragments by chemical modification of HP show that dodecasaccharide and larger fragments had maximal antiproliferative activity (10, 72). Furthermore, Tiozzo et al. (72) demonstrated that the reduction in the molecular weight (MW) of HP is associated with a progressive reduction in the antiproliferative activity. These studies were based on chemically modified HP. In recent years, Joseph et al. (39) attempted to assess the influence of MW, protein core, and GAG chain of native HP on PASMC proliferation. The most potent Upjohn batch of HP was fractionated by dissolving it in water and dialyzing it against water without chemical depolymerization. This was followed by lyophilization of both the dialyze (giving a LMW HP fraction of <3.5 kDa) and the HP fraction retained in the dialysis bag [yielding a high molecular weight (HMW) HP fraction of >3.5 kDa]. The core protein of Upjohn HP was isolated by treatment with heparitinases I and II (25). GAG chains of Upjohn HP were liberated with alkaline borohydride treatment (8). No appreciable difference on the growth inhibition of PASMCs between the LMW and HMW HP fractions was found. The protein core showed no antiprolifera-

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Table 2. Properties of different batches of heparin

<table>
<thead>
<tr>
<th></th>
<th>USP, U/mg</th>
<th>Antiproliferative Activity</th>
<th>Protein Content</th>
<th>Amino Acid</th>
<th>Hexosamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upjohn</td>
<td>140</td>
<td>48</td>
<td>1.56</td>
<td>DTSEGAYFKH</td>
<td>26.3</td>
</tr>
<tr>
<td>Elkins-Sinn</td>
<td>180</td>
<td>12</td>
<td>0.17</td>
<td>SGMYFKH</td>
<td>24.4</td>
</tr>
<tr>
<td>Choay</td>
<td>None</td>
<td>None</td>
<td>1.57</td>
<td>DT(tr)S(tr)EGMFKHR</td>
<td>4.38</td>
</tr>
</tbody>
</table>

USP units of anticoagulant activity are from the manufacturer. Antiproliferative activity is expressed in percent inhibition of bovine pulmonary artery smooth muscle growth in vitro at a concentration of 1.0 μg/ml. Protein content is expressed in percent of total heparin (wt/wt). Amino acids are present in the core protein of heparin. tr, Trace amount. Hexosamine is expressed in percent (wt/wt).

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Fig. 7. A: HP sequences with different sulfonation patterns arbitrarily assigned. Solid arrows, cleavage; broken arrow, no cleavage by heparitinases I and II; *, O-substituent inhibits the cleavage by enzyme treatment. B: cleavage pattern of heparin containing 3-O-sulfonate glucosamine residues by heparitinases I and II. [Reprinted from Garg et al. (25). Copyright 1996, Academic Press.]
Table 3. Substitution pattern of Δ-disaccharide released by heparitinase I and II digestion of different heparins

<table>
<thead>
<tr>
<th>Δ-Disaccharide</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>Upjohn</th>
<th>Elkins-Sinn</th>
<th>Choay</th>
</tr>
</thead>
<tbody>
<tr>
<td>OS</td>
<td>H</td>
<td>Ac</td>
<td>H</td>
<td>0.9</td>
<td>3.9</td>
<td>14.5</td>
</tr>
<tr>
<td>NS</td>
<td>H</td>
<td>SO₃</td>
<td>H</td>
<td>0.3</td>
<td>2.0</td>
<td>3.9</td>
</tr>
<tr>
<td>6S</td>
<td>SO₃</td>
<td>Ac</td>
<td>H</td>
<td>1.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Di (6,N)S</td>
<td>SO₃</td>
<td>SO₃</td>
<td>H</td>
<td>11.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Di (U,N)S</td>
<td>H</td>
<td>SO₃</td>
<td>SO₃</td>
<td>6.3</td>
<td></td>
<td>18.0</td>
</tr>
<tr>
<td>Di (U,6)S</td>
<td>SO₃</td>
<td>Ac</td>
<td>SO₃</td>
<td>1.5</td>
<td></td>
<td>1.5</td>
</tr>
<tr>
<td>Tri (6,U,N)S</td>
<td>SO₃</td>
<td>SO₃</td>
<td>SO₃</td>
<td>66.3</td>
<td></td>
<td>66.0</td>
</tr>
</tbody>
</table>

Results are percent of total Δ-disaccharides released. R₁, R₂, and R₃, substituents on Δ-disaccharides; OS, nonsulfonated; NS, N-sulfonated; 6S, 6-sulfonated; US, uronic acid-sulfonated; Di (6,N)S, 6- and N-disulfonated; Di (U,N)S, uronic acid- and N-disulfonated; Di (U,6)S, uronic acid- and 6-disulfonated; Tri (6,U,N), 6-, uronic acid-, and N-trisulfonated; Ac, acetyl.

The antiproliferative activity of native HA (Fig. 9) became strikingly significant after sulfonation and equaled that of native HP. Because HA, like CS and DS, has a 1→3 linkage between sugars, whereas HP and HS have a 1→4 linkage, the linkages between acidic and basic sugars (Fig. 8, A and E) do not seem to be critical for antiproliferative activity. Acharan sulfate. Neither acharan sulfate nor N-sulfaocharan sulfate had any antiproliferative activity, showing that sulfation alone does not produce antiproliferative activity. CS and DS. Full sulfonation of CS and DS reversed their proliferative effect on PASMCs and produced an antiproliferative effect similar to HP (Fig. 9). Because both CS and DS have a variable sequence that is not sulfonated, this shows that O-sulfonation of both types of sugar residues of the GAG is necessary for the antiproliferative activity. Because both CS and DS GAGs consist of N-acetylgalactosamine residues only, the above data also suggest that N-sulfonated glucosamine residues in HP are replaceable. Furthermore, because all the basic sugar residues are N-acetylated in CS and DS, this suggests that the N-sulfonated basic sugar residues are not critical for antiproliferative activity. Anticoagulant activity. The anticoagulant activity of fully sulfonated HP was significantly reduced compared with the anticoagulant activity of native HP. All the other sulfonated GAGs showed very little anticoagulant activity (24). This demonstrates that the structural determinants of HP for its anticoagulant and antiproliferative activities are unrelated.

In summary, the above studies on the effects of HP and its derivatives on PASMC antiproliferative properties show that 1) 3-O-sulfonate substitution of glucosamine residues is not critical in whole HP for antiproliferative activity, 2) the HMW and LMW of a given HP do not...
affect the potency, 3) the antiproliferative properties of HP reside in the GAG chain and not in the core protein, 4) a certain number of O-sulfonate groups of HP is essential for the full antiproliferative effect of HP, 5) the N-sulfonate group on basic sugar residues is not critical for antiproliferative activity, 6) the basic sugar residues of glucosamine are replaceable with galactosamine residues, 7) the anomeric linkage of acidic and basic sugar
residues is not critical for antiproliferative activity, 8) the commercially available HPs have a varying degree of antiproliferative activity on PASMCS, and 9) the antiproliferative and anticoagulant activities reside in different domains of HP.

CONCLUDING REMARKS

The biosynthesis of HP chains is initiated by the formation of a (GlcAβ1→4GlcNAcα1→4)ₙ polymer (Fig. 3) that is subsequently modified. Various modification reactions are generally incomplete in the sense that only a fraction of the potential substrate residues is utilized at each step. These processes therefore lead to sequence heterogeneity of HP. Functional properties of HP and other proteoglycans depend heavily on their ability to bind receptors. HP binds with receptors in a selective manner. By virtue of this property, HP possesses different types of biological activities. An increase in antiproliferative activity in fully sulfonated HS, DS, CS, and HA shows a potential for the development of one of these derivatives as a therapeutic agent for the treatment of vascular remodeling in the near future.

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REFERENCES


