Investigations into Therapeutic Discovery and Delivery of Heparin-like Glycosaminoglycans

by

Dongfang Liu

SUBMITTED TO THE DIVISION OF BIOENGINEERING AND ENVIRONMENTAL HEALTH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN PHARMACOLOGY AND TOXICOLOGY

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Submitted to the Division of Bioengineering and Environmental Health on May 30, 2001 in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Pharmacology and Toxicology

Abstract

Heparin-like glycosaminoglycans (HLGAGs) are complex polysaccharides found both in extracellular matrix (ECM) and at the cell surface where they, in a sequence-specific manner, bind to and regulate the biological activity of numerous proteins. Due to a lack of effective tools to study structure-function relationship of these complex molecules, the role(s) of HLGAGs in cancer remains largely indirect and poorly understood.

To study the role(s) of HLGAGs in cancer growth, metastasis and angiogenesis, tumor systems were studied using heparinases I and III which have distinct substrate specificities. Studies, focusing on the biochemistry of the heparinase active site, elucidated key interactions important for enzymatic activities, thus enabling the biological studies presented here. In vitro cell culture study with heparinases as tools clearly showed that cell surface HLGAGs are involved in regulating fundamental cellular activities including cell proliferation, invasion and adhesion. Significantly, heparinase I and III demonstrated distinct effect on these cellular activities with heparinase III inhibiting tumor cell proliferation, invasion and adhesion in selected model systems and heparinase I inhibiting these processes.

Further in vivo studies in animal tumor models confirmed and expanded the distinct responses to heparinase I and III treatment. HLGAG fragments generated from heparinase treatment were found to be responsible for the effect of heparinase treatment in tumor models. Moreover, cell surface HLGAGs containing cryptic activatory and inhibitory sequence information were identified and characterized. In contrast to heparinase I-generated HLGAG fragments, heparinase III-generated HLGAG fragments were shown here to inhibit both primary tumor growth and secondary lung metastasis. The inhibition of tumor by heparinase III treatment was attributed to inhibition of specific signaling pathways such as FGF2 signaling.

In an effort to develop a non-invasive and efficient delivery strategy for HLGAG-based therapeutics, HLGAGs were prepared as dry aerosol of defined characteristics for pulmonary inhalation. Pharmacokinetics study revealed efficient absorption for both formulated and unformulated dry aerosolized HLGAGs after pulmonary inhalation. Significantly, pulmonary inhalation of aerosolized HLGAGs was demonstrated to be effective in treating both local respiratory diseases including tumor metastasis and systemic thrombosis.

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PART I
INTRODUCTION

Chapter 1
Motivation and Background

1.1 Motivation

Heparin-like glycosaminoglycans (HLGAGs) are information dense complex polysaccharides that contain sequences of differently modified repeat units that confer the functionality of HLGAGs. HLGAGs ubiquitously exist in the extracellular matrix (ECM) and at the cell surfaces, where they bind specifically to many important proteins including morphogens \(^{1,4}\), growth factors \(^{5-7}\), enzymes \(^{8,9}\), and structural proteins \(^{10}\), thereby regulating key biological processes. One best-studied examples of this is the involvement of HLGAGs in regulating mitogenic signaling by FGF2 in the ECM compartment. This has implications in tumor growth, angiogenesis and development. Despite the intensive research in the field, there is currently no direct evidence for HLGAGs in cancer progression due to the complexity and heterogeneity of HLGAGs. Bacterially derived heparinases I and III that cleave HLGAGs with distinct substrate specificities can be used to study the structure-function relationship of HLGAGs in cancer biology. This thesis research was motivated by the potential therapeutic applications of an improved understanding of HLGAG’s role in tumor biology as well as the development of a novel strategy for delivery of HLGAG-based therapeutics.

The overall goals of this thesis research are to 1) investigate the role of HLGAGs in both primary tumor growth and secondary tumor metastasis; 2) study the potential of delivering HLGAG-based therapeutics as dry aerosol particles via pulmonary inhalation.
1.2 Characteristics of Heparin-like Glycosaminoglycans

1.2.1 Molecular Features of GAGs

Glycosaminoglycans (GAGs) include heparin, heparan sulfate, dermatan sulfate, chondroitin sulfate, keratan sulfate and hyaluronic acid, which are polymers of a disaccharide repeat unit, comprising an uronic acid (U) and a hexosamine (H) \(^{11}\). GAGs are synthesized as homopolymers which may subsequently be modified by N-deacetylation and N-sulfation, eventually followed by C5-epimerization to iduronic acid (I) and 2-O-sulfation of the uronic acid, and 6-O- and 3-O sulfation of the hexosamine \(^{12-15}\). The position and number of sulfate groups attached to the component sugar residues as well as kind of sugar residues provide an enormous number of structural variations to each glycosaminoglycan, by which each glycosaminoglycan is implicated in various physiological functions. The often-extensive sulfation of GAGs in combination with the backbone uronic acids make GAGs among the most acidic biopolymers found \(^{16}\). Table 1.1 provides an overview of the chemical structure and possible modifications for each of the four families of GAGs.

One distinct characteristic of GAGs is the heterogeneity of a GAG preparation with individual chains having different molecular weights and composition. Even among GAG chains that are derived from a single glycosylation site on a single core protein of a single cell line, there is heterogeneity \(^{17-20}\). The differences among the individual chains in a GAG preparation are termed ‘macroheterogeneity. In addition, there can be significant ‘microheterogeneity’ or compositional differences among residues within a single GAG chain \(^{21}\).

The structure of GAG polymers can be described at several levels, somewhat analogous to protein structure \(^{11}\). The ‘primary GAG structure’ is defined by the overall composition of a particular preparation, as well as the sequence of modified repeats, while the ‘secondary structure’ is the spatial appearance of a chain, e.g. repeated helical winding. One could define a ‘tertiary structure’ of GAGs to describe the tendency of chains to fold upon themselves, however, such folding of GAGs has not been described in the literature.

Much evidence points to the conclusion that specificity in binding of GAGs to proteins is correlated to the primary sequence of residues in the chain. However, it is still unknown what role the secondary structure and conformational flexibility of the GAG chain play in these interactions.
Table 1.1. The repeat disaccharide units of GAGs and the organizations of GAGs. Heparan sulfate and dermatan sulfate contain regions of highly sulfated iduronic acid (NS or I) and regions of lower degree of sulfated glucuronic acid (NAc or G).

<table>
<thead>
<tr>
<th>Disaccharide residue</th>
<th>H</th>
<th>U</th>
<th>Modifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin &amp; Heparan sulfate</td>
<td>$H_{NV,NX,3X-(\alpha1,4)U2X-(\alpha1,4)}$</td>
<td>Glucosamine</td>
<td>Iduronic acid or glucuronic acid</td>
</tr>
<tr>
<td>Chondroitin &amp; Dermatan sulfate</td>
<td>$H_{NAC,4X,6X-(\beta1,4)U2X-(\alpha1,3)}$</td>
<td>galactosamine</td>
<td>Iduronic acid or glucuronic acid</td>
</tr>
<tr>
<td>Keratan sulfate</td>
<td>$H_{NAC,6X-(\beta1,3)Gal6X-(\beta1,4)}$</td>
<td>glucosamine</td>
<td>galactose</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>$H_{NAC-(\beta1,4)-G-(\beta1,3)}$</td>
<td>glucosamine</td>
<td>glucuronic acid</td>
</tr>
</tbody>
</table>

![Diagram](image)

Heparan sulfate

Dermatan sulfate

1.2.2 HLGAG Structure

HLGAGs include heparins and heparan sulfates, which are the structurally most diverse glycosaminoglycans. The disaccharide repeat consists of a glucosamine and uronic acid linked by two 1,4 glycosidic bonds (Table 1.1). All chemical modifications possible for GAGs have been found for HLGAGs. HLGAGs are heterogeneous mixtures of closely related polymers. The commercial heparins exhibit molecular weight ranging from 7000-35,000 kDa, whereas heparan sulfate chains may show a similar broad range of molecular weights that are generally two to three fold higher than those of heparins.

Typically, one determines the monosaccharide composition of a polysaccharide by hydrolysis to yield mixtures of monosaccharides and then separation of the monosaccharides by chromatography and quantification of each separated component; this has provided the overall composition of preparation of HLGAGs and other GAGs (Table 1.2).
Table 1.2. Molar composition of GAGs.

<table>
<thead>
<tr>
<th>GAGs</th>
<th>Uronic acid modification</th>
<th>Hexosamine modification</th>
</tr>
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<tr>
<td></td>
<td>IdoUA</td>
<td>2-O S</td>
</tr>
<tr>
<td>Heparin</td>
<td>89-94%</td>
<td>86-92%</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>28-88%</td>
<td>8-81%</td>
</tr>
<tr>
<td>Chondroitin 4-sulfate</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Chondroitin 6-sulfate</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Dermatan sulfate</td>
<td>7-93%</td>
<td>5-12%</td>
</tr>
</tbody>
</table>

N.A. Not applicable; N.D. Not determined. *summarized from references.\textsuperscript{11,21}

Due to the limited availability of heparan sulfate, most structural and sequence studies for HLGAGs have been carried out with heparin. However, due to the compositional difference, information obtained from heparin analysis tends to be biased. The limitations have led to the recent development of sensitive analytical tools for characterization small amounts of HLGAGs. Increased sensitivity of detection of HLGAG oligosaccharides can be achieved by introducing UV chromophores and covalent derivatization using fluorescent or radioactive tags. More recent studies in our laboratory further increased the sensitivity and improved methodology by applying mass spectrometric methods.\textsuperscript{23-25} The use of matrix-assisted laser desorption ionization (MALDI) MS, has made it possible to determine the mass of sulfated oligosaccharides complexed noncovalently with basic peptides to great accuracy (within 1Da). Other powerful approaches include combining separation methodology with mass spectrometric technology (capillary electrophoresis or liquid chromatography).

Despite many shared structural features, heparin and heparan sulfate have distinct features. Several criteria have been proposed to distinguish heparin from heparan sulfate. Some researchers recommended that the term heparin be restricted to HLGAGs in which more than 80% of the GlcN residues are N-sulfated and the number of O-sulfates is greater than the number of N-sulfates; all other related HLGAGs would be referred to as heparan sulfate.\textsuperscript{22} Gallagher and Walker have stated that all heparan sulfates have about 50% of their GlcN residues N-sulfated and have ratios of O-sulfates to N- sulfates of 1 or less.\textsuperscript{26} According to Nader and Dietrich
heparins and heparan sulfates are distinguished by a combination of their physical and biological properties. All heparins are precipitated from tissue extract by 2 M potassium acetate, pH 5.7, at 4 °C; they show motilities in agarose gels similar to those of commercial heparins; they have an average molecular weight of 10,700 to 68,000; they form multiple bands on isoelectric focusing in polyacrylamide gels; and they are digested by heparinase I. Heparan sulfates, however, are soluble in 2 M potassium acetate; do not form multiple bands on isoelectrofocusing; and are minimally degraded by heparinase I. Heparins are composed largely of I2S-HNS,6S and G-HNS,6S whereas heparan sulfate contain only a few percent of the I2S-HNS,6S disaccharide, but contain a much more complex mixture of less highly sulfated disaccharides than heparin. Heparin is synthesized exclusively on the serglycin core protein, and is found stored in intracellular vesicles in mast cells. Heparan sulfate proteoglycans (HSPG) is ubiquitously present on cell surfaces. Fibroblast and hepatocyte proteoglycans each have 46 heparan sulfate chains of 14-20 kDa, while in basement membranes as many as 12 heparan sulfate chains varying from 25-70 kDa can be attached to a single protein anchor. Here, all HLGAGs will be considered as belonging to a family of molecules with continuous range of compositions and properties.

1.2.3 Other GAGs

Chondroitin and dermatan sulfate

Chondroitin and dermatan sulfates arise from a single homogeneous precursor, chondroitin, with the disaccharide repeat unit: [GaK4Aε-(β1,4)-G(β1,3)-ε]11. Subsequent modification involve C5-epimerization of glucuronic acid to iduronic acid, and/or O-sulfation at C2 of uronic acids and C4 or C6 of galactosamine (Table 1.1), which results in complex primary structures of dermatan and chondroitin sulfates, much like for heparin and heparan sulfate. The fraction of uronic acids in dermatan sulfate that are isomerized to iduronic acids varies widely (Table 1.2), whereas all the uronic acids of chondroitin sulfate (CS) are glucuronic acid. Dermatan sulfate (DS) occurs predominantly in small proteoglycans, each containing 2-8 DS chains of 15-55 kDa, while chondroitin sulfates more often are found in large aggregating proteoglycans with 20-100 CS chains of 15-70 kDa.

Specific functional sequences are also present in chondroitin and dermatan sulfates. Heparin cofactor II (HCII) is a thrombin inhibitor, which must bind to dermatan sulfate or
heparin to be active\textsuperscript{29}. By analyzing the activity of various dermatan sulfate hexasaccharides, it was found that only those containing 2-O sulfated uronic acids activated HCII, and those containing three 2-O-sulfated iduronic acids are the most active and constitute 2\% of all hexasaccharides, far more than would be expected if this uncommon residue was randomly distributed\textsuperscript{30, 31}, which is analogous to the function of 3-O sulfation of glucosamine in the AT III-binding pentasaccharide.

**Keratan sulfate**

Keratan sulfate is the only GAG that does not contain uronic acid; therefore, it is not cleavable by the eliminative enzymatic cleavage. Almost all glucosamines and some of galactose units are 6-sulfated in keratan sulfate\textsuperscript{32}. The chains contain a few non-sulfated residues at the reducing end adjacent to the linkage region, followed by a monosulfated region of about 10-12 disaccharides, and a disulfated region of 7-34 disaccharides. These suggest that the enzymes that generated sulfation of keratan sulfate are specific for different sections of chain\textsuperscript{11}.

The branched region and the presence of fucose and sialic acid are characteristics of keratan sulfate, which are also found in the oligosaccharide chains of many glycoproteins\textsuperscript{33}, but not among the other GAGs. In other glycoconjugates, fucose and sialic acid often lead to highly specific binding to proteins\textsuperscript{33}, however, keratan sulfate has not been found to bind specifically to any protein of known biological function.

**Hyaluronic acid**

The disaccharide repeat of hyaluronic acid is $H_{\text{NA}_c}-(\beta 1,4)-G-(\beta 1,3)-$. Therefore, the monosaccharides are the same as in unmodified heparan; however, the glycosidic linkage is similar to those of chondroitin sulfate\textsuperscript{11}. The disaccharide repeat is not modified further, and consequently, hyaluronic acid is homogenous in its primary structure, and does not contain sulfates. Hyaluronic acid chains are considerably larger than those of other GAGs, and can contain up to several thousand disaccharides corresponding to molecular weights of 100-1000 kDa\textsuperscript{11}. As opposed to the other GAGs, hyaluronic acid is not synthesized in the Golgi from a core protein, but rather by an integral plasma membrane synthase, which secretes the nascent chain immediately\textsuperscript{34,35}. Large amount of hyaluronic acid is found in cartilage tissue.
1.3 **Biosynthesis of Heparan Sulfate**

Recent cloning of many biosynthetic enzymes for HLGAGs has improved the understanding of the basis for HLGAG chain structure complexity and function diversity. Heparan sulfate/heparin is synthesized by (a) formation of a region linking the heparan sulfate/heparin chain to protein, (b) generation of the polysaccharide chain, and (c) enzymatic modification of the chain to yield the specific saccharide sequences and structural organization that are responsible for protein binding. The linkage regions of chondroitin sulfate, dermatan sulfate, heparan sulfate (HS), and heparin chains, except keratan sulfate, to the core protein have a common structure, GlcA β1-3 Gal β 1-3Gal β 1-4 Xyl β 1-O-protein.

Biosynthesis of these GAGs is initiated by the transfer of Xyl from UDP-Xyl to a hydroxide group of specific Ser residues in core protein. The transfer of Xyl to core protein is essentially different from that of either GlcNAc or GalNAc to GlcA residue at the non-reducing terminal. The former is a reaction to determine the number of GAG chains in the proteoglycan, and the latter determines the species of GAG. A distinct transferase then adds a single GlcNAc residue to the nonreducing end of the linkage region. This enzyme distinguishes between sites on the core protein intended for glycosylation with HS chains, initiated with GlcNAc, from those sites to be glycosylated with CS chains. This enzyme (GlcNAc Transferase I) has not yet been cloned, and the precise core protein sequence that establishes the HS attachment site is not yet clear. Indeed, initiation with GalNAc and elongation of a CS chain appear to be default pathway.

Alternative addition of GlcA and GlcNAc from their respective UDP-sugar nucleotide precursors by a single HS polymerase forms the repeating 1,4-linked disaccharide HS chain. The length of the HS chains can vary over 10-fold with cell type and core protein, but the chain termination mechanisms are mostly unknown. Once the HS chain is assembled, generally 50-150 disaccharides, the individual saccharide units are subjected to a series of sequential enzymatic modification reactions in which the products of one reaction are substrate for the next. These reactions apparently do not convert all of the available substrate, resulting in substantial sequence diversity in the final chain. The initial modification enzyme is the N-deacetylase/N-sulfotransferase (NDST) that replaces the N-acetyl group of GlcNAc residues, leaving regions of the chain unmodified. The extent of this modification varies among the distinct N-deacetylase/N-sulfotransferase enzymes from rat liver, mouse mastocytoma, and trachea. Then D-glucuronic
acid residues adjacent to GlcNSO₃ residues are epimerized to L-iduronic acid units by glucuronyl C-5 epimerase.

These modified disaccharides will receive the bulk of the subsequent O-sulfations. An iduronosyl 2-O sulftosferase (2-OST) acts, then a glucosaminyl 6-O sulftosferase (6-OST) acts, and the modification reactions are completed with modification of a few residues by glucosaminyl 3-O-sulftosferase (3-OST). The sulftosferase use 3′-phosphodeoxynucleosine 5′-phosphosulfate (PAPS) as a sulfate donor. The PAPS is synthesized from ATP and SO₃⁻ via a single enzyme having both ATP sulfurylase and adenosine 5′ phosphosulfate (APS) kinase activities ⁶⁻⁸. Each of these sulftosferases has multiple isoforms, some of which are tissue specific, share distinct substrate specificities, or both. For example, GlcA-2-SO₃⁻ is a relatively minor component of HS except in brain, suggesting a tissue-specific sulftosferase ⁶⁻⁸.

Biosynthesis of HLGAGs is a concerted process. The mode of interaction of the various biosynthetic enzymes with each other as well as their positioning within the Golgi-complex also need to be elucidated, bearing in mind that substrate recognition by most of the enzymes generally depends on structural modifications introduced in the previous reactions ⁸. The NDS, the C-5 epimerase, the 2-OST, and 6-OST enzymes all appear to be type II membrane-bound proteins while 3-OSTs lack a hydrophobic sequence of sufficient length. NDS-1 and NDS-2 may be located in different Golgi compartments because their N-terminal regions, including the cytoplasmic tail and the transmembrane domain, show little homology, and this region is important for protein retention and localization ⁸.

Other factors that are potentially important include the availability of the sulfate donor, PAPS. The sulfation patterns may change because of modulations of the intra-Golgi PAPS concentration as determined by the Kₘ of the various sulftosferases. However, the overall kinetics of HS assembly, as determined by the membrane-bound state of the biosynthetic machinery, remains poorly understood ⁸.

1.4 HLGAG Degradating Enzymes

1.4.1 Introduction

Up to date, six HLGAG degrading enzymes have been isolated from four different bacteria; Flavobacterium heparinum, Bacillus sp., Bacteroides heparinolyticus, and an unclassified bacterium. The enzymes that have been classified as heparinase I, II and III
from *Flavobacterium heparinum* were cloned and sequenced in our laboratory and will be reviewed here.\(^{42-45}\)

### 1.4.2 Substrate Specificity of Heparinases I, II and III

The catalytic mechanisms of heparinases have been elucidated by a combination of site-directed mutagenesis and analysis of the oligosaccharide degradation products by the mutant enzymes. The degradation product is conveniently monitored on the 232 nm absorbance due to the introduction of the Δ4,5 unsaturated bond to the degradation products.

Heparinases I, II and III are the only HLGAG degrading enzymes that have been well characterized\(^ {21,41}\). Each of these heparinases is specific for a different combination of modifications of the trisaccharide sequence containing the scissile glucosamine-uronic acid linkage (Table 1.3). Heparinase I cleaves \(H_{NS,6X-I2S/G2S-H_{NS,6S}}\) sequences, which are primarily found in the NS regions, while heparinase III cleaves \(H_{NY,6X-I/G-H_{NY,6X}}\) sequences, which are principal components of NAc-regions of HLGAGs (Figure 1.1)\(^ {11}\). Interestingly heparinase II displays unusual enzymatic activity as it is capable of cleaving glycosidic linkages containing either a glucuronic or an iduronic acid residue with no specific substitution requirements, i.e. \(H_{NY,6X-I2X/G2X-H_{NY,6X}}\); as a result that it cleaves both regions of HLGAGs.

These empirical rules for substrate specificity were originally based on the two monosaccharide residues flanking the scissile bond\(^ {46}\), however, more residues are involved in defining the enzyme-substrate interactions. Tetrosaccharides, derived from the AT III-binding pentasaccharide of HLGAGs, were found to be resistant to cleavage with a combination of heparinases I, II and III\(^ {24,25,47}\). NMR studies showed they all had a similar sequence: \(ΔU-H_{NAc,6X-G-H_{NS,6X,3S}}\) where X is either sulfated or unsubstituted\(^ {24,25,47}\). This study showed that residues other than the two flanking a bond are involved in determining the specificity for heparinase cleavage. When an uronic acid is adjacent to a 3-sulfated hexosamine on the reducing side, the glycosidic bond on the non-reducing side of that uronic acid is resistant to cleavage. This implies that the mechanism governing the substrate specificity for heparinase cleavage of HLGAGs involves at least three monosaccharides. More recent research done in our laboratory confirmed and expanded this result.
Figure 1.1. The substrate specificities of heparinases I, II, and III. G: glucuronic acid; I: iduronic acid; U: uronic acid; H: hexosamine.

Table 1.3. Properties of heparinases I, II and III from Flavobacterium heparinum

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>MW (kDa)</th>
<th>$T_{\text{opt.}}$ ($^\circ$C)</th>
<th>$pH_{\text{opt.}}$</th>
<th>Turnover $N^0$ (s$^{-1}$)</th>
<th>Relative Rates</th>
<th>Heparin</th>
<th>Heparan sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparinase I</td>
<td>41.7</td>
<td>35</td>
<td>6.5-7.2</td>
<td>140</td>
<td>100%</td>
<td>20-28%</td>
<td></td>
</tr>
<tr>
<td>Heparinase II</td>
<td>84.1</td>
<td>40</td>
<td>6.9-7.3</td>
<td>27-51</td>
<td>100%</td>
<td>192%</td>
<td></td>
</tr>
<tr>
<td>Heparinase III</td>
<td>70.8</td>
<td>45</td>
<td>7.6</td>
<td>76-166</td>
<td>$\equiv 0$</td>
<td>100%</td>
<td></td>
</tr>
</tbody>
</table>

$T_{\text{opt.}}$ Optimal working temperature. $pH_{\text{opt.}}$ Optimal working pH value. *summarized from references 11,42,43.

1.4.3 Catalytic Mechanism of Eliminative Cleavage

The primary structures of three heparinases showed little homology to other proteins or to each other. By combining biochemical analytical tools and site directed mutagenesis approach, the active site critical amino acids have been largely determined for heparinase I and II. Cys 135, His 203, and lys 199 have been individually identified as the essential amino acids involved in
active site chemistry for heparinase I. In addition, three histidines, His238, His451, and His579 were found to be important for the enzymatic activity of heparinase II. Additionally, in heparinase II, a cysteine, Cys348, which is important in the breakdown of heparin-like regions by heparinase II but not heparan sulfate-like regions, was identified. Heparinase III contains no cysteine residues in its primary amino acid sequence. As mentioned above, biochemical studies of heparinase I and II have shown that cysteine plays an active role in the eliminative cleavage of the HLGAG backbone. Heparinase III does, however, contain twelve histidines. Recent biochemical and site-directed mutagenesis studies have identified histidine 295 and 510 as critical residues that are necessary for the catalytic degradation of heparan sulfate by heparinase III.

Heparinase I and III have two heparin binding consensus sequences, while heparinase II has three. The compliance to consensus sequence in hep III is less stringent compared to hep I, which is consistent with the substrate specificity (less sulfated HS) of hep III. In heparinase I, calcium was found to bind specifically to heparinase I and this binding is required for the full activity of the enzyme. On the other hand, the catalytic activity of hep II and III are not calcium dependent.

Based on the knowledge on heparinase I active site chemistry, a general catalysis mechanism was proposed for eliminative cleavage of HLGAGs by heparinas. The proposed mechanism involves three steps: 1) neutralization of the negative charge on the carboxylate (C6) of the uronic acid, 2) general base abstraction of the C5 proton, and 3) protonation of the leaving glucosamine group. In hep I, the charge neutralization of C6 is thought to be preformed by lysine 199. Cys135 is believed to perform the abstraction of C5 proton and His 203 donate the proton to the leaving group as a result of β elimination.

By applying MS and capillary electrophoresis methods, our laboratory has shown that heparinase I cleaves HLGAGs in an exolytic and processive manner. In an initial step, heparinase I preferentially cleaves exolytically at the nonreducing terminal linkage of the HLGAG chain. In a second step, heparinase I has a strong preference for cleaving the same substrate molecule processively, i.e., to cleave the next site toward the reducing end of the HLGAG chain.
1.5 Modulation of Protein Functions by HLGAGs

1.5.1 Glycosaminoglycan Functions

Cell surface HLGAGs in the form of heparan sulfate proteoglycans (HSPGs) modulate the interactions of extracellular protein ligands with their receptors by forming HLGAG-protein complexes. Table 1.4 listed some proteins that interact with HLGAGs, grouped according to the functions they performed. The ubiquitous HLGAG interactions with protein ligands observed at cell surface and ECM once were considered nonspecific. However, recent research has shown that many HLGAG ligands require GAG sequences of well-defined length and structure (Table 1.4). Two gene families, syndecans and glypicans account for most cell surface HSPGs, both consist of discrete core proteins covalently attached with several HLGAG chains (Figure 1.2). The syndecan family was first discovered, which in mammals contains four gene products with distinctive extracellular domains (ectodomains) and highly conserved short cytoplasmic domains. The glypican family, in contrast to syndecan family, contains six gene products that are

![Figure 1.2](image)

**Figure 1.2.** A. Schematic representation of cell surface HLGAGs in the form of syndecan (left) and glypican (right). The core protein in syndecan is a transmembrane protein with short cytoplasmic domain, and the core protein in glypican is a globular protein rich in cysteins. Typically 2-3 HLGAG chains are attached to the core proteins of either HSPGs. B. HLGAGs involved in FGF signaling. HLGAGs regulate FGF signaling by 1) sequestering FGF in ECM (step 1); 2) transporting FGF to the target receptors (step 2); 3) directly involved in the formation of a ternary signaling complex (steps 4a, 4b, and 4c). Various growth factors such as those listed in the Table 1.4 bind to HLGAGs and the interaction covalently linked to plasma membrane lipid by glycosylphosphatidylinositol (GPI) anchor $^{10,57}$. Various growth factors such as those listed in the Table 1.4 bind to HLGAGs and the interaction
of which with HLGAGs increases their stability and activity \(^{58}\). The short HLGAG fragments may participate in transport of active ligands (Figure 1.2) \(^{59}\). In ECM, HLGAGs may function as storage reservoirs from which growth factors and cytokines can be released in active form to interact with high affinity receptors (Figure 1.2) \(^{60}\). On the cell surface, HLGAGs localize and/or immobilize various ligands for internalization and degradation (lipoprotein lipase (LPL), thrombospondin) \(^{10,61,62}\), or for receptor binding and signal transduction (growth factors). HLGAGs frequently act as coreceptors for protein ligand signaling such as FGFs and integrins (Figures 1.2, 1.3). Additionally, HLGAGs regulate the activity of various proteases and protease inhibitors, particularly in the coagulation cascade \(^{29}\). In addition, HLGAGs are involved in cell adhesion processes through binding to HLGAG binding sequence contained in many structural proteins such as collagens, thrombospondin, fibronectin and vitronectin (Table 1.4) \(^{63}\).

![Diagram](image)

Figure 1.3. HLGAGs as the regulators of protein functions. A. Cell surface HLGAGs as the coreceptor for integrin mediated cell adhesion. B. Cell surface HLGAGs as the coreceptor for FGF signaling.

The specificity of interactions of protein ligands with HLGAGs is determined by multiple factors. Van der Walls and hydrophobic interactions may also be involved \(^{64}\), and presence of conformationally flexible iduronate residues, in particular, favors GAG binding to proteins \(^{65}\). Other factors such as spacing between the protein-binding sites also play a role; \(\gamma\)-interferon dimerization by HLGAGs, which modulates cytokine processing and biological activity, requires GAG chains with two binding sequences separated by a 7-kDa region with low sulfation \(^{66}\). Additional sequences may be required for full biological activity of some ligands; in order to support FGF-2 signal transduction, HS must have both the minimum binding sequence and additional residues that may interact with the FGF receptor \(^{67}\). In turn, heparin-binding proteins
often contain consensus sequences consisting of clusters of basic residues \(^{67,68}\). Lysine, arginine or glutamine are frequently involved in contacts with GAG sulfate and carboxyl groups, and their spacing, sometimes determined by secondary structure, may control the binding specificity\(^{67}\).

Table 1.4. Examples of proteins known to bind HLGAGs\(^a\).

<table>
<thead>
<tr>
<th>Classification of binding proteins</th>
<th>GAG interacting proteins</th>
<th>Selected GAG binding sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECM components and adhesion molecules</td>
<td>Fibronectin, laminin, collagen, vitronectin, L- and P- selectins, PECAM-1, NCAM, thrombospondin, fibrin</td>
<td>Dodecasaccharide composed of repeating I(<em>{2S})(\text{H}</em>{NS, 6S}) for laminin; 7-8 N-sulfated disaccharides containing I(_{1\pm 2S}) for fibronectin.</td>
</tr>
<tr>
<td>Growth factors</td>
<td>FGFs, EGF family, IGF-II, PDGF-AA, TGF-(\beta1,2), VEGF-165, 189, HGF, INF-(\gamma), Wnt/wg, IL-3, GM-CSF</td>
<td>Pentasaccharide containing I(<em>{2S}) however, biological activity require decasaccharide containing I(</em>{2S}) and H(_{6S}) for FGF-2.</td>
</tr>
<tr>
<td>Serpins</td>
<td>AT-III, HCII, protein C inhibitor, nexin-1</td>
<td>H(<em>{\text{NAC}</em>{4S}G-H_{NS, 6S}}), I(<em>{2S})(\text{H}</em>{NS, 6S}), I(<em>{2S})(\text{H}</em>{NA_{4S}})(\text{I}<em>{2S})(\text{H}</em>{NA_{4S}})(\text{I}<em>{2S})(\text{H}</em>{NA_{4S}})(\text{I}<em>{2S}) for ATIII; I(</em>{2S})(\text{H}<em>{NA</em>{4S}G-H_{NS, 6S}}), I(<em>{2S})(\text{H}</em>{NA_{4S}})(\text{I}<em>{2S})(\text{H}</em>{NA_{4S}})(\text{I}<em>{2S})(\text{H}</em>{NA_{4S}})(\text{I}_{2S}) for HCII.</td>
</tr>
<tr>
<td>Chemokines</td>
<td>MIP-1(\beta), RANTES, GRO, IL-8, PF 4</td>
<td>Two hexasaccharide domains containing I(<em>{2S})(\text{H}</em>{NS, 6S}) separated by less than 7 N-sulfated or N-acetylated disaccharides for interleukin-8; 9 kDa sequence with two terminal domains rich in NS and 2S separated by N-acetylated region for PF 4.</td>
</tr>
<tr>
<td>Enzymes</td>
<td>Lipoprotein lipase, superoxide dismutase, elastase</td>
<td>Decasaccharide containing I(<em>{2S})(\text{H}</em>{NS, 6S}).</td>
</tr>
<tr>
<td>Morphogens</td>
<td>Activin, BMP-2,-4, chordin, frizzled-related peptides, sonic hedgehog</td>
<td></td>
</tr>
<tr>
<td>Anti-angiogenic factors</td>
<td>Angiostatin, endostatin</td>
<td></td>
</tr>
<tr>
<td>Coagulation components</td>
<td>Factor Xa, leusserpin, tissue factor pathway inhibitor, thrombin</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) summarized from references \(^{10,11,22,67,69}\).

Since different ligands require distinct HLGAG sequences for binding to GAGs, changes in HLGAG fine structure during development, cell differentiation, or pathological processes will
influence their ligand-binding abilities and, subsequently, the physiological activities of the ligands. Because HLGAG-ligand interactions are complex, it is difficult to dissect individual structure/function relationships. However, cells do modify their enzymatic machinery to synthesize distinct HLGAG chains. In neuronal development, a switch from FGF-1 to FGF-2 activity correlates with synthesis of HLGAG with increased affinity for FGF-2 \(^{70,71}\). In cell culture, syndecan-1 from different cell lines carries HLGAG chains of distinct structure and affinity for collagen, but not FGF-2 \(^{19}\). Cell undergoing tumorigenic process also often modify HLGAG sulfation \(^{72-74}\), leading to altered adhesion properties and different abilities to interact with growth factors.

HLGAG chains are unique among ligand-binding saccharides on the cell surface. Not only is ligand-binding ability determined by their fine structure, but also their action may differ depending on whether they are present as free GAGs, or covalently linked to the core proteins. For example, HLGAGs need to be immobilized on the cell surface to serve as FGF-2 coreceptor (Figure 1.3) \(^{6,7}\). On the other hand, cell regulates growth factor signaling by shedding HLGAG at the cell surface \(^{10}\).

The shedding of the HLGAGs at the cell surface is accomplished by a family of enzymes known collectively as sheddases or secretases. Syndecans is shed rapidly from cell surface by proteolytic cleavage of the core protein \(^{10}\). The site of cleavage for syndecan-1 has been localized to within 9 amino acids adjacent to the extracellular face of the plasma membrane \(^{10,75}\). The syndecan cleaving enzyme is a cell surface zinc metalloproteinase \(^{76}\). It appears that the same enzyme is responsible for cleaving all four syndecans. The glypicans can be shed from the cell surface by the action of glycosylphosphatidylinositol –specific phospholipases \(^{77}\).

The shedding of HSPGs can be regulated by various external stimuli and intracellular signaling pathways. Shedding of syndecans can be enhanced by growth factors \(^{78}\), cellular stress \(^{75}\), and soluble virulence factors of microbial pathogens \(^{76,79}\). These agonists activate specific signaling pathways to stimulate the shedding \(^{76}\). For example, EGF family members act on the shedding mechanism via MAP kinase pathway \(^{75}\). Other signaling pathways such as activation of protein kinases C by phorbol esters appear to regulate shedding as well. These observations suggest that distinct signaling pathways converge to activate the cell surface-associated metalloproteinase that cleaves the syndecans. How they converge to stimulate a common
mechanism is not known. However, available evidence indicates that all regulated syndecan shedding is inhibited by protein tyrosine kinase inhibitors such as genistein and tyrophostin 76.

The shedding of cell surface HLGAGs generates intact, soluble HSPG ectodomain and reduces the amount of cell surface HLGAGs. The shed ectodomains of HSPG retain the ligand binding activities of their cell surface counterparts and can act as soluble biological effectors 76. For example, purified syndecan-1 ectodomains bind tightly to neutrophil elastase and cathepsin G and reduce the affinity of these proteases for their physiological inhibitors, thereby regulate their activities 80. Purified syndecan-1 ectodomains can also function as potent inhibitors of heparin-mediated FGF-2 mitogenicity via the NAc domains of their HS chains 81. Further degradation of the HLGAG chains by HLGAG degrading enzymes such as heparanase and heparinase could result in further regulation of the biological activities of shed HSPG 81.

The reduction of cell surface HLGAGs by shedding or enzymatic degradation can regulate receptor-signaling events. It was shown that treatment of cells with HLGAG degrading enzymes or sulfation-blocking agents (e.g. sodium chlorate) led to the reduced cell proliferation in response to FGF2 stimulation 76,81.

The complex sequence information as well as the regulated expression of HLGAGs in different cell types and tissues enables HLGAGs to participate and modulate diverse biological processes, which are further reviewed in the following sections.

1.5.2 Haemostasis and Thrombosis

Haemostasis is maintained by the clotting system involving both protein factors and platelets. The blood coagulation cascade is activated upon stimulation, resulting in the rapid formation of fibrin clot to prevent the leakage of the vascular system (Figure 1.4). Upon activation, sequential activation of serine proteases, including factor Xa and thrombin (factor IIa), led to the conversion of fibrinogen to fibrin monomers, which further polymerize to form the clot. The clotting system is counterbalanced by several protease inhibitors that keep the clotting event local to the site of injury 29,82.

Thrombi are composed of fibrin and blood cells and may form in any part of the cardiovascular system, including the veins, arteries, heart, and microcirculation. Influenced by the hemodynamic factors, the composition of thrombus in terms of cells and fibrin differ in arterial and venous thrombi 83,84. Arterial thrombi form under conditions of high flow and are
composed mainly of platelet aggregates bound together by thin fibrin strands. In contrast, venous thrombi form in areas of stasis and are composed mainly of red cells with a large amount of interspersed fibrin and relatively few platelets. Thrombi that form in regions of slow to moderate flow are composed of a mixture of red cells, platelets, and fibrin and are known as mixed platelet-fibrin thrombi. When a platelet-rich arterial thrombus becomes occlusive, stasis occurs and the thrombus can propagate as a red stasis thrombus.

**Figure 1.4.** The clotting cascade.

Arterial thrombi usually occur in association with preexisting vascular disease, the most common of which is atherosclerosis; they produce clinical manifestations by inducing tissue ischemia, either by obstructing flow or by embolizing into the distal microcirculation. Activation of blood coagulation as well as platelet activation is important in the pathogenesis of arterial thrombosis. These two fundamental mechanisms of thrombogenesis are closely linked in
vivo, because thrombin, a key clotting enzyme generated by blood coagulation, is a potent platelet activator, and activated platelets augment the coagulation process. Therefore, both anticoagulants and drugs that suppress platelet function are potentially effective in the prevention and treatment of arterial thrombosis.

Venous thrombi usually occur in the lower limbs and are often asymptomatic; however, they can produce acute symptoms if they cause inflammation of the vessel wall, obstruct flow, or embolize into the pulmonary circulation. Activation of blood coagulation is the critical mechanism in pathogenesis of venous thromboembolism, while the role of platelet activation is less important. Therefore, as might be anticipated, anticoagulants are very effective for the prevention and treatment of venous thromboembolism, while drugs that suppress platelet function are of less benefit.

It has been estimated that venous thromboembolism is responsible for more than 300,000 hospital admissions per year in the United States. Pulmonary embolism causes or contributes to death in approximately 12% of patients who are in hospitals and has been estimated to be responsible for 50,000 to 250,000 deaths per year in the United States.

HLGAGs are intricately involved in the clotting cascade. Heparin activates antithrombin III (ATIII) by forming a complex with the protein via a specific pentasaccharide sequence. AT-III binding to the pentasaccharide [GlcN(S/Ac)-GlcA-GlcNS (3-OSO₃)-IdUA (2-OSO₃)-GlcNS (6-OSO₃)] induces a conformational change in the protein that mediates its inhibitory effects (Figure 1.5). The heparin/ATIII complex inactivates a number of coagulation enzymes, including thrombin (IIa) and factors Xa, XIIa, XIa, and IXa; of these, thrombin and factor Xa are most responsive to inhibition (Figure 1.4), and human thrombin is more responsive to inhibition by the heparin/ATIII complex than factor Xa by about one order of magnitude.

Unlike its anti-Xa activity that requires only the AT-III pentasaccharide binding site (Figure 1.5B), heparin's anti-IIa activity is size-dependent, requiring at least 18 saccharide units for the efficient formation of an AT-III, thrombin, and heparin ternary complex (Figure 1.5). Formation of a ternary complex between AT-III, thrombin, and heparin results in inactivation of thrombin. Of specific note, 3-O sulfation of the glucosamine, a mandatory modification within the pentasaccharide sequence, is a rare modification; accordingly only 30% of intact heparin chains contain an intact AT-III binding site. The remaining two thirds of the heparin has minimal anticoagulant activity at therapeutic concentrations, but at high concentrations (greater
than those usually produced clinically) both high- and low-affinity heparin catalyze the antithrombin effect of a second plasma protein cofactor named heparin cofactor II \(^\text{90}\). Activation of HCFI by HLGAGs leads to accelerated inhibition of thrombin by HCFI.

![Diagram A](image)

**Figure 1.5.** The anticoagulation effect of heparin. **A.** Schematic representation of activation AT III by heparin that lead to the formation of a ternary complex inhibiting clotting enzyme such as thrombin. **B.** Illustration of the two different mechanisms during inhibiting of factor Xa and IIa by heparin. Black box represents the specific AT III binding pentasaccharide sequence.

Another mechanism of coagulation inhibition is mediated by thrombomodulin, an endothelial cell surface proteoglycan with a single chondroitin sulfate chain. Thrombomodulin binds thrombin and reduces the activity of thrombin’s activity to fibrinogen and increases the inactivation of IIa by AT III \(^\text{29}\).

Thus, HLGAGs regulate coagulation cascade by four distinct mechanisms; A long HLGAG chain (18 mers or longer containing AT III binding pentasaccharide) serve to promote inhibition of thrombin by AT III, a pentasaccharide containing HLGAG chain serves to catalyze the inhibition of factor Xa by AT III, a GAG chain binds to and inhibits thrombin \(^\text{29}\), and a non-pentasaccharide containing GAG chain binds to HCII and inhibits thrombin.
1.5.3 Extravasation of Circulation Cells

When inflammation occurs in tissue, leukocytes are recruited to the site of inflammation via blood circulation. The process can be divided into distinct stages of cellular events. Signaling molecules released at the inflammatory site induce expression of adhesion molecules on the surface of endothelial cells and leukocytes, leading to arrest of leukocytes in the capillary system close to inflammation site. Initially, one set of recognition molecules forms very elastic bonds that continuously form and break as the leukocyte cells “roll” over the endothelial surface; a second set of molecules eventually form stable bonds between endothelium and the leukocyte to completely stop the motion. Subsequently, leukocytes migrate through the blood vessel wall and its basement membrane and into the site of inflammation. The first set of molecules expressed at cell surface is called selectins, which bind to both sialyl-Lewis carbohydrate structure and heparan sulfate. The second set of interactions primarily involves integrin mediated cell adhesion, which requires HLGAG as the coreceptor\textsuperscript{91,92}.

Both L-selectin and P-selectin bind to heparan sulfate glycosaminoglycan chain on endothelial cells\textsuperscript{93,94}. Recent study has convincingly shown that binding to heparan sulfate is essential for monocytes to adhere to the endothelial cells via L-selectin and subsequent migration through the vessel wall. Additionally, it is found that endothelial cells express a HSPG receptor for the leukocyte L-selectin, and that the HLGAG chains responsible for binding were enriched in the very unusual N-unsubstituted glucosamine\textsuperscript{93,94}. A recent research showed that heparin can interrupt the interaction between the tumor cell surface carbohydrate and the P-selectin expressed on platelet cell surface, a process that promotes the tumor metastasis in the capillary bed of target organ. Consequently, heparin treatment of tumor cells results in reduced metastatic potential\textsuperscript{95}.

HLGAGs are also required for integrin mediated cell adhesion during extravasation of the blood cells. It was shown that HLGAG on adult T cell leukemia cells is involved in chemokine-dependent autocrine stimulation of integrin triggering by immobilizing the chemokines\textsuperscript{91,92}. Thus, HLGAG is important for leukemic cell adhesion to endothelial cells.

1.5.4 Cell Growth and Differentiation- Fibroblast Growth Factors

Cell growth and differentiation are the key cellular functions that regulate all tissue and organ development. As expected, numerous molecules are involved in such highly coordinated
and regulated cellular functions. Many of these molecules, including FGFs, TGF-β1 and -β2, VEGF, CC and CXC chemokines, and various cytokines are known to bind to HLGAGs and their biological activities are modulated due to this interaction (Table 1.4)\textsuperscript{10,67}.

One of the best-studied HLGAG-binding proteins is that of the fibroblast growth factors (FGFs). Fibroblast growth factor (FGF) was originally purified from bovine pituitary gland as a mitogen that could stimulate the growth of NIH3T3 cells\textsuperscript{96}. The discovery that FGF has a high affinity for heparin facilitated its purification and led to the observation that heparin could stabilize FGF from heat and proteolysis. Over the last 25 years 19 FGFs and 4 FGF receptors (FGFRs) have been identified in vertebrates, 2 FGFRs and one FGF have been identified in \textit{Drosophila} (Breathless, \textit{btl}; Heartless, \textit{hlt}; branchless, \textit{bnl}), and one FGF/FGFR pair has been identified in \textit{C. elegans}. All FGFs share an internal core region of similarity with 28 highly conserved, and six invariant amino acid residues. FGFs range in molecular weight from 17 to 34 kDa in vertebrates and up to 84 kDa in \textit{Drosophila}. Structural studies on FGF1 and FGF2 demonstrate that these proteins adopt αβ trefoil structure which contains four-stranded β sheets arranged in a triangular array\textsuperscript{96}. The loop between β strands 10 and 11 contains several basic amino acid residues, which form the primary heparin-binding site on FGF2. Regions thought to be involved in receptor binding include the β8-β9 loop and are distinct from the heparin-binding site.

Rapraeger et al\textsuperscript{7} first demonstrated that in the absence of cell surface HLGAG, FGF-2 interacts poorly with its high affinity FGFR1 and does not activate downstream intracellular signaling. Several models have been proposed to explain the coreceptor role of HLGAGs in FGF signaling. The simplest model is that binding to HLGAGs induces a structural change in the FGF to a form that binds the FGFR with higher affinity. However, this was disproved by the contrary observation that crystallography study showed no detectable structural change in FGF after binding to HLGAGs\textsuperscript{97}. A second model that has been proposed is that HLGAG binding induces dimerization of growth factor which, in turn, promotes dimerization of receptor kinases and facilitates activation by autophosphorylation\textsuperscript{98-101}. This model was supported by some evidence. HLGAG binding to FGF1 and 2 was shown to promote dimerization of the growth factors\textsuperscript{100,101}. The third model proposes that HLGAGs bind to both FGF and FGFR\textsuperscript{102-105}. This model explains that stimulation of FGF2 binding to receptor requires a minimum HLGAG length of 10-12 monomer units, which must contain both 2-O and 6-O sulfates\textsuperscript{106}. HLGAGs can be provided by
either syndecans or glypicans (Figures 1.2 and 1.3). The binding of FGF to FGFR only requires 4-7 monomer units of HLGAGs and the IdaUA 2-O sulfate is required for this binding. It has been suggested that the extra length is required to generate a bifunctional agent that binds FGF and FGFR simultaneously. Binding of FGFR to HLGAG has been demonstrated. Additionally, it has been suggested that the binding of HLGAGs to FGF can stabilize FGF-FGFR complex and increase local FGF concentration.

Although low level of soluble HLGAGs in solution activates FGF signaling, it was observed that high concentration of soluble HLGAGs inhibits FGF activity. A potential explanation to this phenomenon is that the range within which exogenous heparin potentiates FGF2 binding to receptor corresponds to the concentration at which most heparin is adsorbed to the plasma membrane through non-covalent binding to heparin-binding proteins. At higher heparin concentrations these binding sites will be saturated and the amount of soluble heparin will exceed the amount of membrane-associated heparin. Since both bound and soluble heparin bind FGF2, the latter situation would result in sequestration of FGF2 in an inactive form in the medium. This explanation is consistent with the finding that syndecans potentiate FGF2 binding to FGFR when they are associated with the membrane, but inhibit FGF-FGFR interaction when added in soluble form.

The potential to regulate growth factor activity by means of HLGAG-dependent interactions provides an attractive system for fine tuning cellular responses to protein ligands in the ECM. Adjustment of thresholds for cellular response to protein ligands could result from the changes in the level of HLGAGs and their sequence information at the cell surface.

1.5.5 Development

The critical roles of GAGs, especially HLGAGs, in development and specific signaling pathways have recently been demonstrated by the identification of mutations in biosynthetic enzymes for heparan sulfate in Drosophila and mice. Defects in heparan sulfate synthesis, particularly when GAG chains are missing, resulted in severe biological consequences. Mutations in the Drosophila homolog of UDP-glucose dehydrogenase (sugarless), which produces the UDP-GlcA required for GAG synthesis, result in impaired signaling of Wingless, a member of the Wnt family, FGF and Hedgehog, thereby causing very severe phenotypes. Mutations in Drosophila homolog of heparan sulfate N-deacetylase/N-sulfotransferase
(Sulfateless), which catalyzes the initial heparan sulfation step essential for the subsequent modifications, also cause severe impairment of these signaling pathways. In mammalian cells, the latter enzyme is required specifically for the modification of HLGAGs, but not of CS/DS, indicating that HLGAG chains are an absolute requirement for the signaling of these growth factors and morphogens \(^{1-3}\). Notably, mutations in the *Drosophila* homolog of heparan polymerase (*ttv*), which transfers both GlcA and GlcNAc to heparan chains, only affect Hedgehog, not Wingless of FGF, signaling pathways, suggesting a novel and unprecedented level of specificity and regulation for these signaling pathways. *ttv* is a member of the *EXT* gene family (56% identical to human *EXT1* and 26% identical to *EXT2*) and, as mentioned, has been implicated in human HME syndrome \(^{110}\). The function of the EXT1 and EXT proteins as heparan polymerase has recently been elucidated. This together with the finding that overall HLGAG concentration is reduced in *ttv* mutants, suggests that other *Drosophila* *EXT* genes exist. Therefore, Hedgehog signaling is more sensitive to a reduction in HLGAG chains than Wingless and FGF signaling. Alternatively, the specificity of *ttv* to Hedgehog signaling suggests the existence of Hedgehog-specific HLGAG chains. In *ttv* mutants, Wingless and FGF signaling may not be affected because the HLGAG chains, to which these factors bind, are present \(^{111}\).

In addition, it has been reported that mice lacking the heparan sulfate 2-OST exhibit a renal agenesis phenotype \(^{112}\). Moreover, *Drosophila* mutants in the *pipe* gene, also encoding a putative HLGAG 2-OST, show defects in the formation of embryonic dorsal/ventral polarity \(^{112}\). Further, mice carrying a targeted disruption of a single gene encoding one of four identified HLGAG N-deacetylase/N-sulfotransferase genes are unable to synthesize heparin, but produce HLGAG chains \(^{113,114}\). The mice are viable and fertile, but show fewer connective-tissue-type mast cells, in which the absence of heparin results in severe defects in the secretory granules.

1.5.6 HLGAGs as the Co-receptor for Integrin Signaling

Cell adhesion is an essential cellular function, which impinges on many cellular activities including proliferation, migration and differentiation. Integrins, cadherins and selectins are among the major classes of transmembrane adhesion molecules. Over the last decade or so, much has been learned regarding the roles of primary receptor classes such as integrins in cell matrix adhesion \(^{115}\). It is now becoming clear that a second class of cell surface molecules, heparan sulfate proteoglycans, can modify and regulate the type of adhesion mediated by the primary
receptors. HSPGs, especially syndecans, can in addition modify the downstream organization of the cytoskeleton, resulting in distinct adhesive phenotypes.

*In vitro,* trypsin-treated mammary epithelial cells attach to fibronectin-coated surfaces, but the attachment is entirely dependent integrin-mediated adhesion. This is based on the finding that adhesion of trypsin-treated to fibronectin is inhibited by RGD (Arg-Gly-Asp)-containing peptides that block the activity of fibronectin-binding integrins. In contrast, when the cells are prepared by EDTA-mediated release (bivalent cations are required for integrin activity) integrin-blocking peptides no longer inhibit binding to fibronectin. Inhibition of adhesion in this case requires a combination of RGD containing peptide plus soluble heparin or purified syndecan-1 ectodomain. Heparin and the soluble syndecan ectodomain act as competitive inhibitors of binding interactions mediated by HLGAG chains at cell surface. Additionally, treatment of EDTA-released cells with heparinase renders cell adhesion to fibronectin inhibitable by integrin blocking peptides alone. The purified HLGAG-binding domain supports cell attachment, and this is inhibited by soluble heparin but not RGD containing peptide.

The interactions of cells with ECM molecules such as fibronectin result in cell attachment, spreading, and the assembly of focal adhesions and actin stress fibers. Two independent adhesion receptor-mediated signals are required for the assembly of these macromolecular complexes when cell are plated on fibronectin-coated dishes. One signal is mediated through the integrins, which involves the RGD-containing cell-binding domain of fibronectin. The second signal is mediated through cell surface HLGAGs and involves heparin-binding domain of fibronectin. The formation of complete focal adhesions and stress fibers in the context of integrins has been shown to require integrin clustering, integrin occupancy, tyrosine phosphorylation, and cytoskeletal integrity. Integrin-signaling involve the small GTP-binding protein Rho. Early observations identified colocalization of HLGAG with integrin in association with focal adhesions and retention in detergent-resistant cytoskeleton/matrix preparations. In primary fibroblasts, although integrin-ligand interactions are sufficient for cell attachment and spreading, a second signal through interactions of heparin-binding domain of matrix molecules with heparan sulfate proteoglycans was needed for the later stages of focal adhesion and stress fiber formation.
1.5.7 HLGAGs and Cancer Progression

Cancer development is characterized by uncontrolled tumor proliferation and secondary metastasis. For practical purpose, the tumor tissue can be divided into three compartments, namely, the tumor cell compartment, the endothelial cell compartment, and the extracellular matrix compartment. ECM compartment interfaces with both tumor and endothelial cell compartments and regulate the overall development of cellular compartments. HLGAGs along with structural proteins are key components of the cell surface-ECM interface. While collagen-like proteins provide the necessary scaffold for cell attachment and tissue formation, the HLGAG complex polysaccharides fill the scaffold and act as a molecular sponge by specifically binding to and regulating the activities of numerous signaling molecules, such as growth factors and cytokines\(^7,120\). Consequently, HLGAGs actively regulate cell proliferation and migration, the key components for the tumor growth and angiogenesis.

Recent advance in the field has shown that the growth of tumor is strictly angiogenesis dependent; without angiogenesis, tumor will not grow beyond a few millimeters in size\(^121\). Angiogenesis is the sprouting of the new vascular structure from the existing blood vessels. The process of angiogenesis is the result of concerted interplay between protein ligands such as FGFs and endothelial cells. Endothelial cells undergo differentiation, migration, proliferation under the regulation of multiple signals. These signaling molecules can be secreted by tumor cells as a means of inducing tumor angiogenesis.

FGFs play a key role in angiogenesis by inducing proliferation, migration and differentiation of endothelial cells. Additionally, FGFs control the expression of several molecules that are involved in various steps of angiogenesis including collagenase, plasminogen activator and others\(^122,123\). FGF2 signaling has been shown to be a prerequisite for melanoma progression promoting tumor growth in an autocrine fashion, and the interruption of the FGF2 autocrine loop by interfering with either FGF2 or FGF receptor (FGFR) activity results in inhibition of melanoma progression\(^124,125\)\(^126-129\). On the other hand, upregulation of the expression of FGF2 in normal melanocytes result in their malignant transformation\(^124\). Furthermore, FGF2 is a potent and essential angiogenic factor regulating melanoma neovascularization\(^126,130\). Most importantly, specific HLGAG structures are known to bind and modulate FGF2 activity, and there is increasing evidence that, HLGAG sequences, depending on their structure, can either promote or inhibit FGF2 activity\(^131\).
Recent studies have provided much evidence supporting a role for HLGAGs in secondary tumor metastasis. During metastasis, tumor cells in blood circulation first lodge in capillary circulation and induce local coagulation. The formation of fibrin clot protects tumor cells from the host defense and provides substrate upon which tumor cells proliferate and migrate. In vivo studies showed that heparin can potently inhibit tumor metastasis by preventing the tumor cell from inducing local coagulation \(^{132,142}\). Alternatively, recent research provided molecular evidence for heparin's inhibitory effects on tumor metastasis. It was shown that heparin competes with tumor cell surface carbohydrates for binding to P-selectin at the platelet surface, which led to reduced metastatic potential of certain tumor cells \(^{95}\).

Additionally, it was recognized that metastatic tumor cells initially recognize endothelial cell surface HLGAGs \(^{143}\). Following this initial endothelial cell adherence, tumor cells attach via their integrins to the ECM, laminin, fibronectin and collagen with HLGAG as coreceptor. The peptides of laminin and fibronectin, to which HLGAG binds, promoted malignant cell adhesion, \(^{144,145}\). Nanogram quantities of heparin inhibited the adhesion of highly metastatic tumor cells to the structural proteins \(^{146}\). Similarly, HLGAGs, component of liver sinusoidal membranes, are critical for tumor liver metastasis both in vitro and in vivo \(^{73}\). Additionally, it was found that the absolute or relative dominance of HSPG over chondroitin sulfate proteoglycans at the surface of human melanoma cells was a marker of a more malignant phenotype \(^{143}\).

Further, there is much evidence showing profound structural changes for HLGAG during tumor progression. The composition of HLGAGs on cell surface changes depending upon different physiological needs of cells and during malignant transformation. Defined changes in fine structure of cell surface HLGAGs have been documented for a number of human cancers \(^{72,147,148}\). For instance, it has been shown that glypican-1 is drastically upregulated in human pancreatic cancer and the mitogenic effect of FGF-2 and HB-EGF to these cells are abrogated when one removes glypican by either chemical method or antisense targeting \(^{147}\). Also, it was found that 6-O sulfation is increased while 2-O-sulfation and N-sulfation is increased in human colon carcinoma compared to its adenoma precursor, while the length and overall organization is preserved \(^{152}\). In addition, it was shown that the distribution of HSPGs (syndecans, glypican, perlecan) and HLGAG chains in primary human liver tumours is distinguishable to that of normal counterparts \(^{73}\). Furthermore, it was observed that proteoglycans isolated from human breast adenocarcinoma are both quantitatively and qualitatively different.
from normal breast. Adenocarcinoma preparation stimulated endothelial cell proliferation and showed an altered ratio among chondroitin sulfate, dermanan sulfate and heparan sulfate. Thus, aberrant expression of HLGAGs is associated with abnormal cell behavior. By regulating growth factor - receptor interaction, cell adhesion and migration, HLGAGs were suggested to play essential roles in tumor growth, metastasis and angiogenesis.

However, aforementioned studies are mostly indirect observations and the exact roles of HLGAGs in cancer are still poorly understood. The complexity and heterogeneity of HLGAGs and lack of tools to study these molecules have prevented a clear understanding of structure-function relationship for HLGAGs in cancer. Thus, what is required at the present time is direct evidence of the roles of HLGAGs in tumor growth, neovascularization, and metastasis.

1.5.8 Infection

Cell surface HLGAGs appears to be used as a coreceptor by intracellular pathogens; the HLGAGs provide attachment, whereas the other receptor mediates entry of the pathogen into host cells. For example, the initial binding of HSV and other alpha herpes viruses to host cells is mediated by an interaction between viral glycoproteins C and host cell surface HLGAGs. Mutant CHO cells with defective heparan sulfate synthesis, as well as cells pretreated with heparinases to remove cell surface HLGAGs were resistant to infection to CHO cells. Several other pathogens (viruses, bacteria, or parasites) are also shown to use host organism GAGs as adhesion receptors for initial attachments. For bacteria, it was found that *Helicobacter pylori*, which is responsible for gastric ulcer, bind to N-sulfated heparan sulfate specifically, and that cell surface HLGAGs are responsible for the binding of virulent *Borrelia burgdorferi*, which is responsible for the Lyme's disease. Also, it appears that the binding of *Neisseria gonorrhoeae* to cell surface syndecan-4 triggers a signaling cascade that mediates the invasion of this pathogen. Similarly, GAG adhesion is implicated for infection by the parasites such as Trypanosome and *Plasmodium falciparum* (responsible for malaria).

1.5.9 Summary

Aforementioned functionalities of HLGAGs capitalized the diverse biological roles played by HLGAGs. Compared to proteins and genes, however, the full understanding of structure-function relationship of HLGAGs has not been accomplished. This is in part due to the
lack of analytical tools to study these heterogeneous polysaccharides. The potential therapeutic application of HLGAGs in cancer treatment necessitates further research with refined approach in this area.

1.6 Clinical Application of HLGAGs

Despite the diverse biological activities of HLGAGs, there are few polysaccharide drugs currently used in the medicine, among them are unfractionated heparin (UFH) and low molecular weight heparins (LMWHs) \(^{88,159}\). Heparin, discovered by McLean in 1916 \(^{160}\), has been widely used for preventing and treating thrombosis and coagulation for over 65 years. Heparin (UFH and LMWHs) is effective in the prevention and treatment of venous thrombosis and pulmonary embolism, the prevention of mural thrombosis after myocardial infarction, and the treatment of patients with unstable angina and acute myocardial infarction. In addition, heparin treatment has been linked to an improved outcome in cancer patients \(^{143,161}\).

UFH is heterogeneous with respect to molecular size, anticoagulant activity, and pharmacokinetics properties. The molecular weight of UFH ranges from 3,000 to 30,000, with a mean of 13,000 Da (Table 1.5) \(^{162-164}\). The anticoagulant activity of heparin is heterogeneous because only one third of the heparin molecules contain ATIII binding pentasaccharide and because the anticoagulant profile and the clearance of heparin are influenced by the chain length of the molecules, with the higher molecular weight species being cleared from the circulation more rapidly than the lower molecular weight species \(^{88}\). This differential clearance results in an accumulation, in vivo, of the lower molecular weight species, which have a reduced ratio of antithrombin to anti-factor Xa activity. This effect is responsible for the differences observed when the relation between the heparin level and the activated partial thromboplastin time (APTT) is assessed in vivo and in vitro: the lower molecular weight species retained in vivo are measured in the anti-factor Xa heparin assay but have minimal effects on the APTT \(^{88}\).

Due to the rapid metabolism after oral administration, heparin has been exclusively administered as continuous intravenous infusion or subcutaneous injection. After its absorption into the bloodstream, heparin binds to a number of plasma proteins, a phenomenon that contributes to its reduced bioavailability at low doses and the variability of the anticoagulant response to fixed doses of heparin in patients \(^{165}\). Binding of heparin to von Willebrand factor also results in the inhibition of von Willebrand factor-dependent platelet function \(^{166}\). Heparin
also binds to endothelial cells and macrophages, a property that contributes to its complicated pharmacokinetics\textsuperscript{167,168}.

Heparin is eliminated through a combination of a rapid saturable mechanism and a much slower first-order mechanism of clearance\textsuperscript{169-172}. The mechanism of the saturable phase of heparin clearance is thought to be binding of heparin to receptors on endothelial cells and macrophages, where it is internalized and depolymerized\textsuperscript{173-175}. The slower nonsaturable mechanism of heparin clearance is largely mediated by kidney\textsuperscript{88}.

![Graph showing molecular size distribution between UFH and LMWH]

**Figure 1.6.** A comparison of molecular size distribution between UFH and LMWH.

At therapeutic doses a considerable proportion of the administered heparin is cleared through the rapid, saturable, dose-dependent mechanism of clearance\textsuperscript{88,176}. Because of these kinetics, the anticoagulant effect of heparin at therapeutic doses is not linear, although both intensity and duration increase with increasing dose. Therefore the apparent biological half-life of heparin increases from approximately 30 minutes with an intravenous bolus of 25 U/kg to 60 minutes with an intravenous bolus of 100 U/kg to 150 minutes with a bolus of 400 U/kg\textsuperscript{88}.

The bioavailability of UFH is reduced when the drug is administered by subcutaneous injection at low doses (eg, 5000 units every 12 hours) or moderate doses of 12,500 or even 15,000 units every 12 hours\textsuperscript{88,176}. However, at high therapeutic doses of heparin (>35 000 U per 24 hours) the plasma recovery is almost complete\textsuperscript{177}.

In the past two decades also, LMWHs, derived from depolymerization of UFH, have been increasingly used in treatment and prevention of thrombosis for their increased efficacy and reduced side effects. LMWHs are about 1/3 the size of UFH. Like UFH, they are heterogeneous
in size with a molecular weight range of 1800 to 12,000 and a mean molecular weight of 4000 to 5000 Da \(^{88,176}\). Depolymerization of heparin results in a change in its anticoagulant profile, bioavailability and pharmacokinetics, and effects on platelet function and experimental bleeding. A comparison between UFH and LMWHs is summarized in Table 1.5 and Figure 1.6. Due to the reduced chain size and nonspecific binding, LMWHs are predominantly cleared by kidney.

LMWHs apply the same mechanism as the UFH for its anticoagulant and antithrombotic effect. The ATIII binding pentasaccharide was similarly contained in about 1/3 of the LMWH chains. Compared with UFH, which has a ratio of anti-factor Xa to anti-factor IIa activity of approximately 1:1, the various commercial LMWHs have anti-factor Xa to anti-IIa ratios that vary between 4:1 and 2:1, depending on their molecular size distribution \(^{88,176}\).

As a result of reduced chain length, LMWHs bind much less avidly to heparin-binding proteins than does UFH, a property that contributes to their superior bioavailability at low doses and their more predictable anticoagulant response \(^{178}\). They also do not bind to endothelial cells in culture, a property that could account for their longer plasma half-life \(^{167,168,179}\). When
compared on a gravimetric basis, LMWHs are slightly less effective antithrombotic agents than heparin but produce much less bleeding in models used to measure blood loss from a standardized injury.\textsuperscript{88,176,180}

As a result of improved clinical profile, LMWHs become increasingly important in treatment and prevention of thrombosis. LMWHs have a longer plasma half-life and a more predictable anticoagulant response than UFH, so they can be administered once daily and without laboratory monitoring. In animal models they produce less bleeding than heparin for an equivalent antithrombotic effect, so patients can be treated with doses of LMWHs that produce a higher anti-factor Xa level than UFH without safety being compromised\textsuperscript{88,176,180}.

Much evidence has also shown a beneficial effect in cancer patients in association with the use of heparin. Heparin is often used in the practice of oncology to control the hypercoagulability that commonly accompanies malignancy\textsuperscript{181,182}. It is effective in preventing thrombosis after cancer surgery\textsuperscript{183} or thrombosis of venous access catheters used to administer chemotherapy\textsuperscript{161}. More importantly, much evidence has shown that heparin also contain anticancer activities that seem unrelated to its anticoagulant properties. Heparin may directly inhibit tumor progression\textsuperscript{161}. More than a decade ago, Folkman et al showed that heparin in conjunction with cortisone is a potent angiogenesis inhibitor\textsuperscript{184}; angiogenesis is known to be essential for tumor growth and metastasis\textsuperscript{184-188}. Heparin was also shown to inhibit both primary tumor growth and secondary metastasis in a number of animal models\textsuperscript{132,135-138,161,189}. Goerner first reported inhibitory effects of heparin on tumor growth in animal tumor model in 1930\textsuperscript{190}. Subsequent studies also showed inhibitory effect of heparin toward tumor metastasis in a number of animal tumor models\textsuperscript{132,133,135-138,140-142,189}. Clinical study in human patients also revealed protective effect of heparin in non-small cell lung cancer when combined with chemotherapy\textsuperscript{191}. LMWHs appear to have a more pronounced anti-tumor activity and fewer side effects compared to that of UFH\textsuperscript{161}.

The inhibitory activity of heparin toward cancer can be attributed to the diverse biological roles played by HLGAGs. HLGAGs can regulate tumor growth by modulating angiogenesis\textsuperscript{5,184}, the activities of tumor-derived autocrine growth factors and paracrine factors\textsuperscript{96,101,107,131,192} as well as cancer-related coagulation events\textsuperscript{135,136,189,191,193}.

It is realized that most evidence supporting a beneficial effect of heparin treatment in cancer came from a few studies where heparin is merely used as anticoagulant in supplement to
that of chemotherapy. Additional prospective randomized trials are needed to define the efficacy of heparin in cancer treatment and provide the mechanism for the action of heparin in cancer treatment.

1.7 Specific Aims

HLGAGs are the most information dense biopolymers known to regulate a variety of biological functions including tumor progression, angiogenesis and coagulation. The HLGAG polysaccharide consists of a disaccharide repeat unit of a glucosamine and an uronic acid; it can be modified at five different sites to create chemically and functionally distinct sequences in the polymer. The complexity and chemical heterogeneity of HLGAGs coupled with the lack of effective tools to study these polysaccharides has seriously limited investigations into the roles of HLGAGs in tumor growth and metastasis. Up to date, the roles for HLGAGs in tumor progression have been limited to indirect observations due to lack of tools. Thus, what is required at the present time is direct evidence of the roles of HLGAGs in tumor growth, angiogenesis, and metastasis. In an effort to develop tools for study HLGAGS, our laboratory has developed three HLGAG-degrading enzymes, heparinases (hep) I, II, and III. Hep I, II, and III are bacterially derived HLGAG degrading enzymes with distinct substrate specificities that can be used to analyze the composition and sequence of individual GAG chains for study of structural basis of HLGAG function in tumor development, and for potential therapeutic application of these enzymes.

A clear delineation of the roles of HLGAGs in tumor growth and metastasis will allow a new paradigm of how the extracellular polysaccharides modulate tumor growth and metastasis, identify a novel therapeutic target by providing a framework towards the development of HLGAG-based novel anti-cancer molecules.

HLGAG-based therapeutics such as heparin are known to subject to rapid metabolism in gastro-intestinal tract; parenteral injection (i.v. and s.c.) has been the only administration choice. Lung alveoli contain the largest absorption surface in the body. Thin barrier between the rich capillary bed and the alveolar space coupled with the very high blood flow rate makes alveoli of lungs one of the most desirable drug delivery sites. Pulmonary delivery of protein, peptides and small molecules have generated promising results. However, due to the hydrophilicity and high molecular weight, pulmonary delivery of HLGAGs has not been successful. The
extensive research recently in the HLGAG field promised more therapeutic application of HLGAGs in medicine. Pulmonary delivery of HLGAGs, if successful, has the advantages of noninvasive, convenient, and safer administration compared to current injection method. In addition, delivery of HLGAGs directly to lung can potentially achieve high local concentration, improve effectiveness, and minimize systemic side effects associated with injection method.

This thesis research is meant to study the roles of HLGAGs in tumor biology, identify potential therapeutic values of HLGAGs in cancer, as well as develop a novel strategy for delivering HLGAG-based therapeutics to expand and facilitate the application of HLGAGs in medicine. To improve the usefulness of heparinase I in biomedical research and application, this thesis research began with a complete biochemical study on the calcium binding sites in heparinase I.
PART II ENZYMEOLOGY OF HEPARINASE I

Chapter 2. Mapping and Characterization of Calcium Binding Sites in Heparinase I

Summary

This chapter reports the results of biochemical studies on calcium binding sites within heparinase I. Heparinase I is a useful enzyme in both basic research and medicine. Despite major progress in delineating the catalytic mechanism of heparinase I, a complete catalytic mechanism remains unknown. Calcium was known to bind specifically to heparinase I, and this binding is required for maximal activity of heparinase I \(^{48,50,54}\). However, the sites and the amino acids within heparinase I protein that are involved in calcium binding were not identified. To improve the usefulness of heparinase I in medicine and research, it is necessary to have a complete understanding on the enzymology of heparinase I. In this thesis research, by combining biochemical tools with site-directed mutagenesis study, the calcium binding sites and specific amino acid residues that coordinate calcium were identified. Tryptic mapping study located two calcium-binding sites within heparinase I with CB-1 in proximity of heparin binding site and CB-2 in the C-terminus of heparinase I. Site-directed mutagenesis study identified D212, G213, T216 in CB-1 and N375, Y379 and E382 in CB-2 as critical amino acids involved in calcium binding. All mutant enzymes were fully characterized for their enzymatic kinetics, substrate and calcium binding affinities. Significantly, S377A mutant was found to become calcium-independent. A mechanistic discussion for both CB-1 and CB-2 was provided. These results resulted two publications and one patent protection \(^{54,198,199}\).
2.1 Introduction

Heparinase I is proven to be useful in both biological research and clinical applications\textsuperscript{200-203}. Heparinase I, together with heparinases II and III, are powerful tools in study structure-function relationship for HLGAGs in biology. Recent development in HLGAG sequencing technology is based on the distinct substrate specificities of heparinases I, II, and III\textsuperscript{25,204}. Since its discovery, several potential applications for heparinase I in medicine are being explored. Injection of heparinase I is currently being evaluated in clinical trials for systemic neutralization of heparin following thoracic surgery, in which the patient receives artificial lung-heart support and consequently is heparinized to avoid coagulation in the extracorporeal system\textsuperscript{205}. This research has led to the development of an extracorporeal bioreactor, where heparin is continuously infused as the patient's blood enters the extracorporeal device (dialysis, lung-heart machine, or other), and the plasma heparin is subsequently being degraded by passing the blood through the bioreactor system with immobilized heparinase I, as it is returned to the patient\textsuperscript{201-203}. In addition, heparinas are used in production of low-molecular weight heparin such as Tinzaparine\textsuperscript{206}, which is widely used clinically as antithrombotic agents. Further clinical applications of GAG-degrading enzymes include a heparin assay based on quantitative enzymatic degradation that has been approved by the FDA\textsuperscript{207,208}, and the use of heparinase to inhibit neovascularization\textsuperscript{5}.

In order to understand the catalytic mechanism and further improve the usefulness of hep I. Our group has completed extensive biochemical and mutagenesis study for heparinase I to identify the critical amino acids involved in catalysis. Our laboratory showed that Cys\textsuperscript{135}, His\textsuperscript{203} and Lys\textsuperscript{199} are important in the catalytic mechanism of heparinase I (Figure 2.1)\textsuperscript{48,49,209}. Through extensive site-directed mutagenesis studies, other positively charged residues (Lys\textsuperscript{132} and Lys\textsuperscript{198}) that provide the necessary microenvironment for heparinase I catalysis were identified\textsuperscript{209}. Recent studies of the heparin degradation by heparinase I revealed a processive nature of catalytic mechanism, in which heparin is predominantly degraded exolytically from the non-reducing end.
AQKKSGNIPYRVNQADSARKAHNDKWVAVGINKPYALQYDDKLPRNGKPSYREFLKAEDHSLEGYA
AGETKGRTELSSYATTHDFKKFPPSVYQNAQLKLTVVHYGKGICEQGSSRSYTFSVYIDSPDFA
WHGAPRSAVATPEGEIKLTSEEFLALYDEMIFKKHIAHDKVKEKKDKDGKITVYAGKPGNWKVVEQGGYP
199 203
TLAPIFSKGMFYIKANSRQWLTDDAOAHNNAPENSVMEKPSSYEKTSTIAAYKMPFAQFPRDCWITFVDVA
IDWTEYGEKANTILKPGKVMMITYTKENKQQAHVNVQGSEILGRDDGYFPGIYRVNHSTVPVTYNLS
GYSSTAR

Figure 2.1. The protein sequence of heparinase I. Bolded letters represent the critical amino acid residues for enzyme catalysis.

Figure 2.2. Enzymatic activity of heparinase I as a function of calcium concentration.

Despite a good knowledge on the catalytic chemistry for heparinase I, a thorough understanding of heparinase I catalysis is still not established. This is in part due to an incomplete understanding on the roles of calcium on the enzymology of heparinase I. As stated, it is clearly known that calcium binds specifically to heparinase I and the binding is required for the full activity of heparinase I. A complete activity for heparinase I is achieved at a calcium concentration of 5 mM or higher (Figure 2.2). By using the fluorescent calcium analog terbium,
our laboratory found that heparinase I binds calcium specifically. In addition, it was shown that Woodward's Reagent K (WRK) and EDC can inactivate heparinase I, while preincubation with calcium and/or heparin can afford protection from inactivation. Thus it is clear that the interaction between heparinase I and calcium is essential for the proper functioning of the enzyme.

The goals of this research is to locate the calcium binding site(s) within hep I and clearly identify the individual amino acids coordinating the calcium ion. The success of this research will allow a more efficient application of hep I and provide molecular basis for engineering novel heparinase I with improved usefulness.

2.2 Mapping Calcium Binding Sites in Heparinase I

To locate the calcium binding sites in heparinase I, tryptic mapping studies were preformed. Tryptic digests of heparinase I were performed essentially as described previously. 16 μg of heparinase I was added with 4 mM WRK; the sample was allowed to incubate for 30 min at room temperature. A 10-fold excess of glycine methyl ester was added to quench the reaction. The enzyme was then denatured in 50 μl of 8 M urea and 0.4 M ammonium carbonate, reduced with 5 mM dithiothreitol at 65 °C, cooled to room temperature, and alkylated with 10 mM iodoacetamide for 15 min. The reaction was quenched with water by bringing the total reaction volume to 200 μl. To the above reaction was added 4% (w/w) trypsin, and the digestion was carried out at 37 °C for 24 h. The proteolytic reaction was terminated by freezing at −20 °C. The digest was separated using gradient reverse-phase HPLC (2-80% acetonitrile in 0.1% trifluoroacetic acid for 120 min). Tryptic peptides were monitored at 210, 277, and 320 nm and collected. Based on the peptide peaks monitored at 320 nm, five peaks were collected and sequenced using an on-line Model 120 phenylthiohydantoin-derivative analyzer (Biopolymers Laboratory, Center for Cancer Research, Massachusetts Institute of Technology). To determine whether preincubation with calcium protected the enzyme from WRK modification, heparinase I was first incubated with 100 mM CaCl₂ at room temperature. Heparinase I digests in the absence of WRK modification were included as controls. WRK modification of specific amino acid
Figure 2.3. Mapping study of calcium binding sites in heparinase I. A. HPLC analysis of trypsin digested heparinase I observed at 232 nm. B. HPLC analysis of heparinase I after trypsin digestion of WRK modified heparinase I. C. Sequences of modified peptides. Five peaks were isolated. The first, eluting at 52 min (td 52), has the sequence (K) AIIDNK. The second (td 54) and fourth (td 59) peaks both were different modification products of the sequence (K) NIAHDKEKKDK. The third peak (td 56) has the sequence (R) VNVQADSAK. The last peak (td 95) has the sequence (K) FGIYRVGNSTVPVTYNL. SGYSETAR.

residues forms covalent adducts that are stable to proteolytic mapping. In the presence of a suitable nucleophile, such as glycine methyl ester, the WRK-carboxylate adduct absorbs in the near-UV region (280-320 nm) 54. Therefore, the tryptic map of heparinase I was monitored at 210, 280, and 320 nm, and peaks with absorbance higher than that of the controls were collected. Figure 2.3 shows the HPLC profile of the tryptic digest of heparinase I incubated with WRK monitored at 210 and 320 nm. In the chromatogram Figure 2.3B, the peptides eluting at 52 (td
52), 54 (td 54), 56.5 (td 56), 59 (td 59), and 95 min (td 95) were sequenced. The complete sequences of the peptides are shown in the figure legend, and their positions within the primary amino acid sequence of heparinase I are shown in Figure 2.3C. The late eluting peak (td 95) was found to correspond to the C-terminal region of heparinase I. Two of the four clustered peaks (td 54 and td 59) were found to correspond to a region of the protein that overlapped with the primary heparin-binding site of heparinase I. The other two peptides (td 52 and td 56) were found to be small peptides in the N-terminal region of heparinase I, both of which contain aspartate residues.

This mapping study identified two potential calcium binding sites, CB-1 and CB-2 that conform to the canonical calcium binding motif EF-hand. As part of the primary heparin-binding site in heparinase I (residues 196-221), CB-1 extends from residues 207-219 (Figure 2.3C). CB-2 is located at the C-terminus of heparinase I, and spans residues 373-384. It suggests that either one or both calcium binding sites play an important role in calcium binding and/or enzyme activity. To address the functions of each calcium-binding site, site-directed mutagenesis study of CB-1 and 2 was performed.

2.3 Site-directed Mutagenesis Study of Calcium Binding Sites in Hep I

2.3.1 Method

Mutagenesis, Expression and Purification of r-Heparinase I

To choose the amino acids for site-directed mutagenesis study, CB-1 and 2 were aligned with the canonical calcium binding sequence, the EF-hand (Table 2.1). CB-1 spans the residues 207-219 and CB-2 includes residues 373-384. Amino acids in CB-1 and CB-2 containing oxygen side chains and/or conform to the EF-hand consensus sequence were selected for mutation (Figure 2.4). In CB-1, single mutants D210A, D212A, E207A, G213A and T216A were created first. To determine whether the combined mutation of calcium coordinating residues produces a more pronounced effect on calcium binding and enzyme activity, the double
**Table 2.1.** Alignment of putative calcium binding sites amino acids (a.a.) in the heparinase I with EF-hand consensus sequence. The canonical EF-hand consists of a $\alpha$-helix, a loop wrapped around the Ca$^{++}$ ion, and a second $\alpha$-helix. The numbers given in the table represent the positions of amino acids in the loop region. X, Y, Z, -X, -Y and -Z represent amino acid residues that coordinate the Ca$^{++}$ ion $^{210,211}$. CB-1 and CB-2 of heparinase I are aligned against the consensus sequence for calcium binding EF-hands, which includes the helix-loop-helix motif $^{210,211}$. The coordinates for binding of the calcium are indicated with bold letters. Ile at position 8 is seen only in CB-1.

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Mutants D210A/D212A, E207A/D210A, E207A/D212A, D212A/T216A, and G213A/T216A were constructed and expressed. Finally, triple mutants (E207A/D210A/D212A, D212A/G213A/T216A) of all putative calcium coordinating residues were also generated. In CB-2, double mutants G378A/Y379A and E381A/T382A were first expressed. If double mutations had an effect on the catalytic activity, the residues were then individually changed to alanines to examine the possibility of one of the residues having a dominant effect on enzyme activity. Finally, Thr$^{373}$, Asn$^{375}$ and Ser$^{377}$ were individually changed to alanine (Figure 2.4).

The mutations were introduced via 12 cycle PCR, as described previously by the method of Higuchi $^{212}$. All the mutant genes were cloned into pET-15b and were sequenced to verify the mutations as described previously $^{48}$. The recombinant and mutant heparinase I were expressed without the putative *F. heparinum* leader sequence; i.e. as a construct (-L r-heparinase I) that reads Met-Gln22-Gln23-... $^{45}$. To facilitate purification, the heparinase I gene was expressed
using the pET-15b system (Novagen, WI). This construct has a poly-histidine tag and a thrombin cleavage site in a 21 amino acid N-terminal leader sequence.

**CB-1**

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**CB-2**

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</tr>
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<td>E381</td>
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Double mutants: G378A/Y379A, E381A/T382A.

**Figure 2.4.** Schematic representation of the various heparinase I mutations completed for this study. Two putative calcium binding consensus sequences (CB-1 and CB-2) were chosen as targets for mutagenesis study.

All mutant recombinant heparinases (r-heparinases) were constructed in the pET15b expression system (Novagen, WI), expressed and purified in the BL21 (DE3) host as described previously. SDS-PAGE was carried out using 12% gels and a Mini Protean II apparatus, and stained with the Silver Stain Plus kit. The level of protein expression for all the r-heparinases was identical in the BL21 (DE3) host. Kinetic parameters $K_{cat}$ and $K_m$ were determined for all the mutants as described previously. Fluorescence competition study was conducted in selected mutants to determine their calcium binding property. The product profiles of heparin degradation were characterized using anion-exchange HPLC (POROS) to resolve the oligosaccharide products as described.
Characterization of the mutant hep I

The kinetic study of hep I and mutant enzymes was completed by using the UV 232 nm assay as described previously. Briefly, the enzyme activity was directly measured from the increase in absorbance at 232 nm as a function of time. All assays were performed at 30 °C. When measuring the enzyme activity as a function of heparin concentration, heparin concentrations were varied from 0 to 4 mg/ml at a fixed calcium concentration of 5 mM (100 mM MOPS buffer/5 mM calcium acetate, pH 7.0). The data was then fit to a non-linear equation to determine the $K_{cat}$ and $K_m$ of heparinases I and its mutants. Heparinase mutant activity was also investigated as a function of calcium concentration ranging from 0 to 10 mM. This data was also fit to a non-linear function to determine $K_{0.5}$, i.e., the calcium concentration at which half of the maximum enzyme activity was observed. Activity is expressed as IU-μmol product formed/min using ε = 3800 M⁻¹cm⁻¹.

The oligosaccharide degradation products of mutant hep I were characterized by HPLC analysis. Briefly, heparin (4 mg/ml) was incubated with -L r-heparinase I and mutant enzymes in 100 mM MOPS, 5 mM calcium acetate buffer, pH 7.0, for 18 h. The reaction was terminated and subjected to anion-exchange HPLC to resolve the oligosaccharide products, as described.

To examine the affinity of mutant heparinase I for its substrate heparin, heparin-POROS chromatography was performed. The heparin-POROS chromatography study of heparinases I was essentially carried out following the procedure described previously. Briefly, about 30-40 μg of -L r-heparinase I and the various mutant enzymes were injected into a heparin POROS (4.6 mm X 100) column (PerSeptive BioSystems, Framingham, MA) connected to BioCAD system (PerSeptive BioSystems). Proteins were eluted using a linear gradient of 0-1 M NaCl in 10 min (10 mM Tris, 1 mM EDTA, pH 7.0) and monitored at 210 nm. EDTA was added to chelate any calcium ions that may have been present in the buffers.

To directly study the calcium binding affinity of the mutant hep I, fluorescence competition assay was completed for selected mutants. To accomplish this, the ability of heparinase I and mutants to compete for free calcium with the calcium-chelating fluorescence probe rhod-5N was examined. The fluorescence probe rhod-5N was dissolved and diluted in buffer A (10 mM MOPS and 100 mM KCl, pH 6.5). Buffer A and the water used in the study were run through a Chelex Resin column to remove trace amounts of calcium. In the absence of calcium, rhod-5N is not fluorescent, however upon binding calcium rhod-5N is fluorescent with
an emission $\lambda_{\text{max}}$ of 576 nm. Before titration, rhod-5N and heparinase I were added to a quartz cuvette such that the final concentration of rhod-5N in the cuvette was 0.3 $\mu$M and heparinase I is 3 $\mu$M. To this solution was added aliquots of a calcium solution (20 mM) which had been previously equilibrated with 0.3 $\mu$M rhod-5N and 3 $\mu$M heparinase I. The stock was added such that the calcium concentration in the cuvette was 50, 100, 200, 500, 1000, 5000 $\mu$M. After allowing the solution to come to equilibrium, the sample was scanned from 560 nm to 600 nm with the excitation wavelength fixed at 561 nm. The plot of $I/I_{\text{max}}$ versus [Ca$^{2+}$] was fitted to a non-linear equation to determine the apparent $K_d$ ($K_{d'}$) value. Thus, $K_d'$ values represent the apparent dissociation constants for the fluorescence probe rhod-5N $^{198}$. Control samples without heparinase I were included in the study.

### 2.3.2 Results

**Mutagenesis study of CB-1.**

Table 2.2 lists the kinetic parameters obtained for wild-type $r$-heparinase I and all the mutant enzymes. E207A had no effect on the enzyme activity with a $K_{\text{cat}}$ value of 92 sec$^{-1}$ (Table 2.2). D210A and D212A had a moderate effect on enzyme activity ($K_{\text{cat}}$ values of 74 and 65 sec$^{-1}$, respectively). G213A and T216A affected enzyme activity significantly with about a 3- and a 2-fold decrease in $K_{\text{cat}}$ values, respectively, compared to that of wild-type $r$-heparinase I (Table 2.2). Furthermore, the double mutants E207A/D210A and E207A/D212A showed no further reduction in enzyme activity as compared to the corresponding single mutants D210A and D212A. Similarly, the triple mutant E207A/D210A/D212A was not significantly different from double mutant D210A/D212A (Table 2.2). This suggests that Glu$^{207}$ individually or jointly is not essential for enzyme activity. Since Asp$^{210}$, Asp$^{212}$, Gly$^{213}$, and Thr$^{216}$ individually affected enzyme activity, the effect of combined mutations of these amino acids was further studied. Double mutations D210A/D212A and G213A/T216A produced a more pronounced reduction in enzyme activity (approximately a 5-fold reduction in $K_{\text{cat}}$ values) than any of the individual mutations (Table 2.2). The triple mutant D212A/G213A/T216A also decreased the enzyme activity approximately 5-fold. However, no further reduction in enzyme activity was observed for the double mutant D212A/T216A as compared to the T216A single mutation.
Table 2.2. Kinetic constants and salt elution concentrations of wild-type r-heparinase I and mutant heparinases I. Kcat refers to the number of product molecules catalyzed by heparinases I per second; Km refers to the Km value for the heparin substrate; \( K_{0.5} \) refers to the calcium concentration required for the half-maximal activity of heparinases I; NaCl refers to the concentration at which the individual enzyme is eluted from heparin-affinity column.

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<th>( K_{cat} )</th>
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<th>( K_{0.5} )</th>
<th>NaCl</th>
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<td></td>
<td>sec(^{-1})</td>
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<td>( \mu M )</td>
<td>mM</td>
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<tr>
<td>-L</td>
<td>92</td>
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All mutations in CB-1 resulted in increases in \( K_{0.5} \) values, which represent the calcium concentrations at which half of the maximum enzyme activity was observed (Table 2.2). Furthermore, there is a strong correlation between loss of enzymatic activity and an increase in the \( K_{0.5} \) (Figure 2.5). These results suggest that CB-1 mutants lower the enzymatic activity of heparinase I primarily through lowering its calcium affinity. Moreover, this result is consistent with what is seen upon comparison of the exhaustive heparin digests of recombinant heparinase I and the mutants E207A/D212A, E207A/D210A/D212A, and D212A/G213A/T216A. Mutations in CB-1 do not affect the product profile of heparinase I but simply slow the enzyme's catalytic turnover rate (Figure 2.6A, B, C, D).

53
Mutagenesis study of CB-2.

CB-2 was initially screened by targeting key amino acids within the site and creating double mutations. For the G378A/Y379A double mutant, the enzyme activity was reduced significantly (10 fold reduction in $K_{cat}$). A similar effect was observed for the double mutant E381A/T382A (the $K_{cat}$ was decreased by 9 fold). Since the joint alteration of Gly$^{378}$ and Tys$^{379}$ as well as Glu$^{381}$ and Thr$^{382}$ affected heparinase I activity drastically, the effect of individually altering these residues to alanines was investigated to examine whether one mutation had a more pronounced effect than the other on heparinase I activity. When Gly$^{378}$ and Thr$^{382}$ were individually changed to alanines, their $K_{cat}$ values were decreased only by about half (Table 2.2). However, the Y379A and E381A single mutations decreased enzyme activity ($K_{cat}$) by about 10 fold, suggesting these residues are important for calcium binding and/or heparinase I activity. Since Thr$^{373}$, Asn$^{375}$ and Ser$^{377}$ also have oxygen-containing side chains and conform to the EF-hand motif consensus sequence (Table 2.1), the effect of individually changing these amino acids on heparinase I activity was studied. For the T373A and S377A mutants, no significant decrease in enzyme activity was observed (Table 2.2). The N375A mutation decreased heparinase I activity ($K_{cat}$) by more than 9 fold.

Unlike CB-1 mutants, all CB-2 mutants showed decreased $K_{0.5}$ values (Table 2.2). These results were puzzling in light of what was seen for CB-1. Therefore additional experiments were completed to understand the role of CB-2 in calcium binding and heparinase I enzymatic activity (see below and see discussion).

First, the exhaustive digest product profiles for the mutants N375A, S377A, G378A/Y379A, and E381A/T382A were analyzed, which showed a marked effect on activity. The digest profiles were similar to that of -L r-heparinase I, but, unlike CB-1 mutants, there was a decrease in the proportion of the major products (essentially di- and tetra-saccharides) and a greater fraction of digestion fragments larger than hexasaccharides (Figure 2.7A, B, C, D). The presence of digestion fragments larger than hexasaccharide argues for a role for CB-2 in the processivity of heparinase I (see discussion).
**Figure 2.5.** Plot of $K_{0.5}$ versus $K_{cat}$ for CB-1 mutants. The data on the single, double, and triple mutants in CB-1, including both the turnover rate ($K_{cat}$) and the calcium concentration at which half maximal activity is observed ($K_{cat}$) are derived from Table 2.2. A linear regression was run on this data and yielded an $r^2$ value of 0.69.

**Figure 2.6.** Anion-exchange HPLC separation of oligosaccharides. Heparin (4 mg/ml) was incubated with -L and mutant heparinas I in 100 mM MOPS buffer, 5 mM calcium acetate, pH 7.0, for 18 h as described. The reaction was then subjected to anion-exchange HPLC using a POROS Q/M (4.6 x 100 mm) column (PerSeptive BioSystems, MA) with a salt gradient of 0-2 M NaCl in 10 min and monitored at 232 nm. A shows the product profile of heparin degradation by wild-type $\tau$-heparinase I; B shows the product profile of heparin degradation by E207A/D210A; C shows the product profile of heparin degradation by E207A/D210A/D212A; D shows the product profile of heparin degradation by D212A/G213A/T216A.
Heparin Affinity Chromatography.

In light of the differences in the heparin digest product profiles of CB-1 versus CB-2 mutants, it is necessary to understand whether calcium plays a role in heparin binding to heparinase I. In this case, mutations that affected calcium binding might also affect the binding of the substrate heparin to the enzyme. Previous study showed that, in the absence of calcium, native heparinase I from *F. heparinum* binds a heparin-POROS column and can be eluted at a salt concentration around 500 mM. This technique has also been used to investigate whether mutations in heparinase I affect binding of heparin to the enzyme. Thus, heparin-POROS chromatography was used in this study to investigate whether the mutant enzymes had altered salt (NaCl) elution profiles. As shown in Table 2.2, wild-type r-heparinase I eluted at a salt concentration of about 482 mM. For CB-1, all mutants except for Gly²¹³ and Thr²¹⁶ eluted at a higher salt concentration. Interestingly, a direct correlation was observed between the salt concentration eluted and loss of negative charge of carboxyl groups in both CB-1 and CB-2 as shown in Figure 2.8. This trend in the elution profile is expected since the interaction between heparinase I and heparin includes both a non-specific ionic component and a specific heparin-heparinase component. For the non-specific ionic component, neutralizing repulsive negative charges through mutagenesis creates a more favorable interaction while converting a neutral amino acid to alanine results in no effect. Most of the CB-2 mutants (T373A, N375A, S377A, G378A, Y379A, T382A and G378/Y379A), which involve no charge neutralization, eluted at salt concentrations comparable to wild-type r-heparinase I (Table 2.2). Therefore, all of the mutations involved in this study resulted in little to no change to the ability of heparinase I to bind heparin specifically, consistent with the earlier observation that heparinase I is able to bind heparin in the absence of calcium. Together with the observation that all mutant enzymes retained enzymatic activities to a various extent, these results suggest the structure of the enzyme was unlikely to be perturbed upon site-specific mutagenesis, which justifies the comparison of kinetic parameters between mutant enzymes and wild-type r-heparinase I.
Figure 2.7. Anion-exchange HPLC separation of oligosaccharides. Heparin (4 mg/ml) was incubated with mutant heparinases I in 100 mM MOPS buffer, 5 mM calcium acetate, pH 7.0, for 18 h as described. The reaction was then subjected to anion-exchange HPLC using a POROS Q/M (4.6 mm x 100 mm) column (PerSeptive BioSystems, MA) with a salt gradient of 0-2 M NaCl in 10 min and monitored at 232 nm. A shows the product profile of heparin degradation by N375A; B shows the product profile of heparin degradation by S377A; C shows the product profile of heparin degradation by G378A/Y379A. D shows the product profile of heparin degradation by E381A/T382A.

Figure 2.8. A plot of salt elution concentrations for heparinases I as a function of the number of negative charges neutralized. D210A, E207A, and D212A had one negative charge neutralized. D210A/D212A, E207A/D210A, and D210/D212A each had two negative charges neutralized. E207A/D210A/D212A had three negative charges neutralized.
Fluorescence Competition.

The results of the kinetic analysis of CB-1 and CB-2 mutants raised the question of whether mutations in CB-2 mediate their effect through decreasing heparinase's I affinity for calcium. To test this hypothesis directly (i.e., not indirectly through measuring enzyme activity as a function of calcium concentration), the ability of heparinase I and selected mutants to bind calcium was tested using a fluorescence titration assay. Thus, the rationale for the fluorescence competition study is, using the calcium-chelating probe rhod-5N, to determine whether mutations in CB-1 or CB-2 affect the ability of heparinase I to bind calcium. In this study, heparinase I is expected to bind and compete for calcium with the fluorescence probe rhod-5N, and this competition is expected to lower the apparent affinity of rhod-5N for calcium. This decrease is reflected in an increase in the apparent $K_d$ values. Those mutants (E207A/D210A/D212A and D212A/G213A/T216A in CB-1, N375A, E381A, and G378A/Y379A in CB-2) that showed significant decrease in enzyme activity were chosen for fluorescence competition study. **Table 2.3** shows the data derived from the fluorescence competition study. As expected, wild type -L r-heparinase I binds calcium and competes the calcium off the fluorescence probe, resulting in a significantly increased $K_d$ value. Triple mutants in CB-1 both have a diminished ability to compete for calcium and only lead to a minor increase in $K_d'$. The calcium binding ability of the CB-2 mutants lies between the wild type enzyme and the CB-1 mutants, suggesting a moderate reduction in the calcium binding ability. This result confirmed that both CB-1 and CB-2 are involved in calcium binding, albeit differently (see discussion).

**Table 2.3.** Fluorescence competition study with wild-type r-heparinase I and mutant heparinase I. $K_d$ equals to 117 μM in the absence of enzyme. A lower $K_d$ value means less binding of calcium by heparinase I.

<table>
<thead>
<tr>
<th>Enzyme tested</th>
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<td>E381A</td>
<td>175</td>
</tr>
<tr>
<td>G378A/Y379A</td>
<td>136</td>
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</table>
2.3.3 Discussion

The data generated from this research confirms that both calcium binding sites in heparinase I are involved in calcium binding and enzyme activity, albeit differently. Site directed mutagenesis studies in CB-1 identified Asp$^{210, 212}$, Gly$^{213}$, and Thr$^{216}$ as important residues in calcium binding and enzyme activity; kinetic studies showed that these corresponding mutants, individual or combined, decreased the $K_{cat}$ value for the degradation of heparin by heparinase I and increased the $K_{0.5}$ value for calcium (Table 2.2). In addition, examining the $K_{cat}$ and the $K_{0.5}$ values for CB-1 mutants indicates an inverse correlation between the two values, suggesting that these mutants are lowering the enzyme activity of heparinase I through decreasing the binding of calcium to the enzyme. Fluorescence studies further confirmed that mutation of these residues to alanines led to a decreased calcium binding affinity in the CB-1 mutant enzymes (Table 2.3). Thus, taken together, these studies show that CB-1 binds calcium and that mutations in CB-1 mediate their affect, either entirely or in part, through decreasing heparinase's I affinity for calcium.

One important observation of this study is that the latter half of CB-1 (including Gly$^{213}$ and Thr$^{216}$) appears to be more important than the first half of CB-1 (Glu$^{207}$, Asp$^{210, 212}$) in calcium binding and enzyme activity. G213A, T216A, and G213A/T216A gave $K_{cat}$ values of 28, 50, and 19 sec$^{-1}$ compared to 92, 74, 65 and 25 sec$^{-1}$ obtained from E207A, D210A, D212A, and E207A/D210A/D212A. A similar trend was observed when comparing the $K_{0.5}$ values for calcium binding between these same mutant enzymes (Table 2.2).

A second observation derived from this study is that both CB-1 and CB-2 are involved in calcium binding; however, CB-2 plays a more prominent role in heparinase I activity. As shown in the fluorescence competition study, mutations in both CB-1 and CB-2 decreased the calcium binding affinity of heparinase I. On the other hand, mutations in CB-2 (N375A, Y379A, E381A, G378A/Y379A, and E381A/T382A) decreased enzyme activity drastically ($K_{cat}$ values were decreased by about 10 fold), while none of the mutations in CB-1 reduced enzyme activity by greater than 5 fold. Together with the $K_{0.5}$ data, these results indicate that mutations in CB-2 exerts a more pronounced effect on heparinase I, and thereby, the residues in CB-2 mediate their effect on heparinase I activity through interactions that are more complex than CB-1.
If CB-2 is involved in calcium binding to heparinase I, why does a mutation in CB-2 result in a decrease in $K_{0.5}$? One interpretation of these results is that both CB-1 and CB-2 bind calcium; CB-1, which conforms more readily to the consensus calcium chelating motif, is a high affinity site. On the other hand, CB-2, which conforms less readily to the consensus calcium chelating motif, is presumably a lower affinity calcium binding site (Table 2.1). Mutations in CB-1 result in a CB-1 site with decreased affinity for calcium; however selected mutations in CB-2 completely eliminate its ability to bind calcium. In this case, the $K_{0.5}$ for CB-2 mutants is reflective of calcium binding to site 1.

This interpretation is consistent with three observations. First, in the fluorescence competition experiments, mutations in CB-2 resulted in an enzyme that is more like wild type heparinase I as compared to CB-1 mutants in competing calcium away from rhod-5N. This points to the fact that CB-1 binds calcium better than CB-2. Second, there is very little variation in the $K_{0.5}$ of the CB-2 mutants, consistent with the hypothesis that any mutation in CB-2 eliminates CB-2’s ability to bind calcium. Also, the $K_{0.5}$ value for CB-2 mutants, ~50-90 μM, is probably reflective of the affinity of calcium for CB-1. Finally, the heparin binding properties of CB-2 mutants suggests that other possible effects, including unfolding of the protein, are not likely to occur here. Together, these results, coupled with the biochemical studies of the previous paper, point to two sites in heparinase I that bind calcium, a high affinity site (CB-1) and a lower affinity site (CB-2).

2.4 Role of Calcium in Heparinase Catalysis

While both CB-1 and CB-2 of heparinase I are shown here to be essential for maximal heparinase I activity, it remains to be seen by what mechanism CB-1 and CB-2 mediate a role for calcium in heparinase activity. Since calcium has been best known for its ability to induce a conformational change upon binding to many calcium binding proteins. One would expect calcium to play a similar role in heparinase I. However, CD studies with heparinase I in the presence and absence of calcium revealed no conformational change. In addition, based on the previous study that posited the importance of a ternary complex between heparin, heparinase I, and calcium in terms of the enzyme activity of heparinase I, it would seem likely that calcium plays a more fundamental role in the enzymatic mechanism of heparinase I.
There are potentially two roles that calcium binding can play in the enzymatic activity of heparinase I. One possibility is that calcium is directly involved in the active site chemistry of heparinase I. Based on a general mechanism proposed by Gerlt and Gassman for enzyme catalyzed β-elimination reactions of carboxylic acid-containing substrates, a cation, such as calcium, can interact directly with the anionic carboxylate group of the substrate and sufficiently decrease the pKa of the carbon acid such that abstraction of the α-proton can be achieved by a basic amino acid of the enzyme.

It was therefore proposed that in heparinase I, Cys\textsuperscript{135} exists as a thiolate anion in the active site pocket of heparinase I and initiates the abstraction of the C-5 proton of uronate. In addition, His\textsuperscript{203} and Lys\textsuperscript{199} have been shown to play a role in catalysis. In extending the above mentioned theory of a concerted acid-base catalysis to the β-elimination reaction of heparinase I, the abstraction of the C-5 proton by the base, presumed to be the thiolate anion of Cys\textsuperscript{135}, would require a general acid catalyst acting on the carboxyl group. Ca\textsuperscript{++} could potentially satisfy such a requirement by acting as a Lewis acid (Figure 2.9). Alternatively, Lys\textsuperscript{199} could act as an acid catalyst to protonate the carbonyl oxygen in carboxyl group, and Ca\textsuperscript{++} could act to stabilize either deprotonated Lys\textsuperscript{199} or Cys\textsuperscript{135} (Figure 2.9). The polarization of the carboxyl group by Ca\textsuperscript{++} or Lys\textsuperscript{199} would acidify the α-proton at C-5 and facilitate the abstraction by Cys\textsuperscript{135} of heparinase I. Histidine could act as a second acidic catalyst to protonate the leaving β-substituent (Figure 2.9). If calcium plays such a role in heparinase I, then CB-1 would seem to be an attractive candidate in chelating calcium for this purpose. Its proximity to the heparin-binding site of heparinase I as well as to the catalytically critical Lys\textsuperscript{199} or His\textsuperscript{203} and the direct correlation between loss of enzyme activity and loss of enzyme binding upon mutation of CB-1, strongly argue that CB-1 may play a direct role in the enzymatic turnover of heparinase I.
An additional possible role for calcium is that it can be involved in the processivity of heparinase I and/or modulate the exolytic preference of heparinase I. Recently, our group has shown that heparinase I is an exolytic/processive enzyme. Thus, heparinase I preferentially cleaves at the non-reducing end of the HLGAG substrate, but, instead of releasing the products, heparinase I retains the product, moving to the next scissile bond and clipping the substrate. In this case, the mode of action of heparinase I is characterized as processive, starting from the non-reducing end and clipping the substrate towards the reducing end. Instead of being directly involved in the catalytic mechanism as outlined above, calcium might be involved in “shuttling” the HLGAG substrate through the active site core of heparinase I without release of the substrate. If calcium is involved in this activity, then the presence of a low affinity calcium binding site, such as CB-2 in heparinase I, would be essential for proper shuttling of the substrate. Such a role for CB-2 is also consistent with the observation that loss of activity is associated with the
production of higher order digestion fragments such as would be formed if the processivity of heparinase I is affected (Figure 2.7A, B, C, D). Experiments are in progress to address this issue especially whether calcium is involved in the processivity of heparinase I.

2.5. **Summary**

In summary, this study confirms that calcium binds to heparinase I. Further, the research presented here mapped the calcium binding sites, CB1 and CB2, and identified key residues in CB-1 and CB-2 that are critical for proper functioning of heparinase I. Within CB-1 the latter half of the calcium-chelating sequence, including Gly²¹³, and Thr²¹⁶, are more critical for activity. Mutation of selected residues within this sequence affects both enzyme activity and calcium binding activity by heparinase I. Mutations within the second binding site, CB-2, have a greater effect on the enzymatic activity of heparinase I arguing for a more pronounced role for CB-2 as compared with CB-1 in the enzymatic activity of heparinase I. It remains to be seen if calcium plays a direct role in the catalytic reaction mechanism or in the exolytic/processive preference of heparinase I, or both.
PART III
HLGAGS and Tumor Progression
Chapter 3

*In vitro* Roles of HLGAGs in Cancer Biology

**Summary**

This chapter describes the findings on the roles of HLGAGs in tumor progression. *In vitro* cell culture studies were first performed to examine, at cell level, what potential roles are played by cell surface HLGAGs during tumor development. In the adhesion study, heparinase III, but not heparinase I, significantly inhibited the adhesion of tumor cells to the collagen and laminin coated plates. Similar results were observed in the invasion assay where heparinase III prevented the invasion of tumor cells through ‘basement membrane’ while hcp I had minimal effect. The inhibitory effect of heparinase III on tumor cell functions was further observed in a proliferation assay. Taken together this research has demonstrated a direct role for HLGAGs in the pathophysiology of tumor cells, providing foundation for further probing into the *in vivo* roles of HLGAGs in tumor progression.
3.1 Introduction

Many, if not most, of the molecular events associated with tumor growth, neovascularization, and metastasis are influenced by interactions between cells and their extracellular matrix (ECM). HLGAGs along with structural proteins are key components of the cell surface-ECM interface. While collagen-like proteins provide the necessary scaffold for cell attachment and tissue formation, the HLGAG complex polysaccharides fill the scaffold and act as a molecular sponge by specifically binding to and regulating the activities of numerous signaling molecules, such as growth factors and cytokines\textsuperscript{10}. Important progress has been made in understanding the diverse roles of collagen (and related proteins) and enzymes (viz. collagenases) that degrade the proteinaceous component of the ECM in regulating tumor growth and metastasis\textsuperscript{185,188}. Up to date, much has been learned about the important roles of HLGAGs in mediating protein ligand interactions in various cell culture studies. However, partly due to a lack of effective tools, a structure-function study on HLGAGs' role in regulating tumor cell activities has been lacking. The diversified structural characteristics and information density of HLGAGs\textsuperscript{20} are expected to allow them to regulate tumor pathophysiology in multiple ways. Thus, this part of the thesis research set out to establish a direct evidence for the roles of HLGAGs in fundamental tumor cell activities such as invasion, proliferation and adhesion. Heparinases I and III with distinct substrate specificities were applied as powerful tools in this study.

3.2 Methods

Cell adhesion assay

24-well plates were coated at 10 μg/well with collagen I, fibronectin, and laminin. 80% confluent cells were detached and resuspended in serum free medium containing 1 mg/ml BSA to make single cell suspension. 0.3 ml of the cell suspension, containing 8000 cells, was loaded onto the plates at time zero. Experimental groups were incubated with hep I and III or inactivated hep I and III (100 nM each) at 37°C for 30 min before loading onto the plates. Plates were observed under microscope at 0, 10, 30, 60, 90, and 120 minutes after plating. The degree of attachment was graded as described previously\textsuperscript{216}. Briefly, 0 indicates no attachment; 1 indicates most cells have attached but vibrate upon motion of media; 2 indicates all cells have
attached and no vibration can be observed; 3 indicates some cells spreading; 4 indicates most cells are spreading.

**Invasion assay for B16BL6 and PC-3 cells treated with hep I and hep III**

25 μg of Matrigel was deposited onto inserts with an 8 μm pore size according to manufacture’s instruction. Single cell suspensions of $1 \times 10^6$ cells/ml were prepared as described. Treatment groups were incubated with 200 nM heparinases I and III at 37 °C for 30 min. 0.1 ml of the cell suspension, containing $1 \times 10^5$ cells in serum free medium, was loaded onto the inserts and conditioned medium was used as a chemoattractant for cell migration (Figure 3.1). Plates were incubated at 37 °C with 5% CO₂ for 20-24 hours. Then, the inserts were fixed and stained with hematoxylin. The unmigrated cells on top of the membrane were removed by swabbing with cotton applicators. The number of migrated cells was counted with an inverted-light microscope at 200 x magnification. At least 8 randomly chosen fields were counted for each insert.

![Flow chart and schematic](image)

**Figure 3.1.** Flow chart (left) and schematic depiction (right) of invasion study.

**Tumor cell proliferation assay**

0.5 ml cell suspension (4000 cells per well) was seeded on to a 24-well plate on day 1. Starvation of cells with serum free MEMα medium starts on day 2. On day 3, cells were washed with serum free medium before adding hep I and III (100 nm) to the treatment group. Treatment with hep I and III was repeated on day 4 and 5. On day 6, cell proliferation was assayed by acid phosphatase assay²¹⁷.
Western blotting study of HLGAG’s role in FGF signaling in B16BL.6 melanoma cells

B16BL.6 cells in 10 cm culture dishes were serum starved for 48 hours before stimulation with 50 ng/ml FGF2. Cells were stimulated for 20 minutes before whole cell lysates were prepared with 1 ml modified RIPA buffer containing various enzyme inhibitors (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, leupeptin, and pepstatin, 1 mM activated Na3VO4, 1 mM NaF). Protein concentration in the lysate was determined using the Bio-Rad protein assay kit (BioRad) and adjusted accordingly for electrophoresis analysis. For the heparinase treated groups, cells were treated with either hep I or hep III (200 nM) for 30 min at 37°C prior to addition of FGF2. The immunoblot was probed with anti-Erk-1, 2 or anti-phospho-Erk-1, 2 antibody (New England Biolabs; MA) and detected by anti-rabbit IgG conjugated to HRP using SuperSignal West Pico Chemiluminescent substrate (Pierce, IL).

3.3 Results and Discussions

Extensive in vitro experiments were completed using defined cell culture models to examine the effects of HLGAG degradation on tumor progression. HLGAGs at the cell surface have been shown to be involved in cell adhesion process in various non-tumor models. HLGAGs co-localize with integrin in the focal adhesion site and blocking either of the components led to incomplete adhesion with lack of the formation of focal adhesion. It has been shown that HLGAGs mediate cell adhesion by binding to specific binding sequences in the structural proteins including fibronectin, laminin and collagen. The binding sequences for HLGAGs are often located at different sites from those integrin binding sites (RGD containing sequences). The interaction of both HLGAGs and integrin to the same structural protein such as fibronectin triggers the cell signaling processes that lead to formation of focal adhesion and cell adhesion. This concerted interactions between HLGAGs and integrin signaling is analogous to HLGAG-FGFR relationship. In both cases, HLGAGs work as the coreceptor for the primary ligand receptor. Heparinases I and III are HLGAG degrading enzymes that are able to digest cell surface HLGAGs and leave behind clipped HLGAG chains with different structural characteristics. Consistent with this notion, treatment of fibroblast cells with HLGAG degrading enzymes showed reduced cell adhesion.
In the present study, the distinct substrate specificities for heparinases I and III allow for a direct study on how sequence information of cell surface HLGAGs regulate cell adhesion in tumor cells. Heparinase III treatment of B16 melanoma cells significantly inhibited the adhesion of B16 cells to collagen and laminin coated plates (Figure 3.2). The adhesion of B16 cells to fibronectin coated plates, however, was not affected by treatment of cells with either heparinase I or III. Further, this inhibition seems to be specific to hep III treatment since hep I treatment of B16 cells showed little effect on B16 cell adhesion to structural proteins. In heparinase III treated cells, the inhibition of adhesion is more pronounced at spreading stage. On the other hand, control group treated with inactivated heparinase III showed little effect on the adhesion of B16 cells to the structural proteins tested. The results of adhesion study argue that both the length and sequence information of HLGAG chains are important in mediating cell adhesion process. Treatment with both enzymes reduced the length of the HLGAG chains at cell surface. However, what left behind at the cell surface as the ‘clipped’ HLGAGs and those HLGAG fragments released into the solution are different for heparinase I and heparinase III treatment. Heparinase I clips at highly sulfated region of HLGAG chains while heparinase III acts at unsulfated regions of HLGAG chains. As a result, heparinase I treatment of cell surface HLGAGs would release less sulfated HLGAG fragments and leave behind unsulfated HLGAG chain at the cell surface, while the opposite is true for heparinase III treatment of cells. The free HLGAG fragments can further compete with the cell surface HLGAGs for binding to structural proteins, thereby regulate the adhesion process of cells. However, the results of this study suggest that the regulation of tumor cell adhesion is sequence dependent. This interesting observation was further tested in different *in vitro* assays and eventually in animal models.

To test whether the regulation of cell adhesion by heparinase treatment is cell type dependent, human prostate cancer cell line (PC-3) was treated similarly. Consistent with the observation made with B16 cells, heparinase III treatment of PC-3 cells also resulted in inhibition of adhesion to laminin coated plates (Table 3.2). Unlike B16 cells, however, heparinase treatment of PC-3 cells showed little effect on PC-3 cell adhesion to either collagen or fibronectin coated plates (Table 3.1; 3.3).

In the invasion assay, hep III treatment of B16BL6 cells rendered these cells less likely to invade a Matrigel support (Figure 3.3). Conversely, hep I treatment made the cells more invasive as compared to a PBS control. Comparable results were observed when PC3 cells were
treated with heparinases I and III in the invasion assay. The result of invasion assay agrees with that of adhesion assay, but in addition invasion assay saw the opposite effect as a result of heparinase I and III treatment of tumor cells. The observed change again is deemed to result from the clipped HLGAG coat at the cell surface as well as the HLGAG fragments released by heparinase treatment. However, unlike the adhesion assay, signaling molecules other than RGD-containing molecules are likely to be involved. The conditioned medium is rich in growth factors and cytokines, some of which act as chemoattractant for tumor cell invasion. As mentioned in the introduction part of this thesis, HLGAGs bind and regulate many of protein ligands including growth factors and cytokines (Table 1.4). The HLGAG fragments released from tumor cell surface by heparinase treatment may modulate the activities of these protein ligands by either promoting or inhibiting their signaling depending on the sequence information encoded in the specific HLGAG fragments. This might be the reason for distinct phenotypic change observed as a result of differential degradation of HLGAG chains.

In both adhesion and invasion assays, heparinase III treatment seems to be consistently inhibitory toward tumor cell activities. To further examine this effect of heparinase III, proliferation assay was performed. Consistent with its effect observed in the previous assays, hep III treatment inhibited the proliferation of B16 cells (Figure 3.4). The inhibition of tumor cell proliferation by heparinase treatment is rather expected. As stated earlier, HLGAGs are mandatory coreceptor for several growth factors including FGFs. Additionally, B16 cells are known to produce large amount of FGFs to sustain rapid proliferation in an autocrine fashion. The interruption of this autocrine loop in B16 cells was shown to inhibit tumor progression in B16 melanoma. It is conceivable that heparinases III treatment of cells can abolish the coreceptor function of the cell surface HLGAGs.

To corroborate the above findings at a molecular level, the influence of heparinase treatment on intracellular signaling pathways was examined in vitro. Since FGF-2, a heparin-binding growth factor, is well known to be abundantly secreted by B16BL6 melanoma cells forming an autocrine circuit stimulating spontaneous B16BL6 cell growth in vitro, FGF-2 mediated processes is specifically studied in the in vitro system. In addition, since it is well known that FGF-2 primarily activates cell proliferation through the MAPK pathway, the level of Erk-1 and 2 activation (phosphorylation) was examined. Of note is the fact that many oncogenes encode proteins transmitting mitogenic signals via the MAPK pathway. Consistent with the
previous observation, hep I treatment potentiated Erk-1 and 2 activation by FGF-2, while hep III treatment appeared to have alleviated the activation of Erk-1, 2 (Figure 3.5), suggesting that hep III treatment of B16 cells can potentially produce a tumor suppressor effect.

Collagen I

![Collagen I graph]

Laminin

![Laminin graph]

**Fig. 3.2.** Adhesion assay for B16BL6 cells to plates coated with different ECM proteins. 24-well plates were coated at 10 μg/well with collagen I or laminin (Becton Dickson, MA) according to manufacture’s instruction. A. Adhesion assay for B16BL6 cells using a collagen I coated 24-well plate. B. Adhesion assay for B16BL6 cells on laminin coated plates. Cells were prepared and treated as described in the methods. It was noted that heparinase treatment of B16 cells had no effect on cell’s adhesion to fibronectin coated plates.

**Table 3.1.** The evaluation of cell adhesion onto fibronectin coated plates by PC-3 cell.

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<th>Time (min)</th>
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Table 3.2. The evaluation of cell adhesion onto laminin coated plates by PC-3 cells.

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Table 3.3. The evaluation of cell adhesion to collagen I coated plates by PC-3 cells.

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Figure 3.3. Invasion assay for (A) B16BL6 and (B) PC-3 cells treated with hep I and hep III. The invasion assay was performed as described. * indicates p < 0.05 (Mann-Whitney test).
Figure 3.4. The effects of hep III treatment on B16BL6 cell proliferation. * indicates $p < 0.05$ (student t-test).

- FGF2  +FGF2  +FGF2  +FGF2  
+Hep 1 +Hep III

Phospho-Erk-1, 2

Erk-1, 2

Figure 3.5. Level of total and phosphorylated Erk-1, 2 in FGF-2 stimulated, heparinase treated B16BL6 cells. The immunoblotting was performed as described in the method section. It is noted that hep I treatment of cells potentiated the activation of Erk-1, 2 by FGF2 treatment, while hep III treatment of the cells showed the opposite effect.

3.4 Implications

These results clearly showed a direct role for HLGAGs in tumor cell functions. More importantly, the study suggested that sequences generated from heparinase III treatment of tumor cells contain either tumor inhibitory or tumor promoting information. These observations also
support the paradigm that HLGAG fragments can play an intricate role in modulating tumor growth and metastasis. In the following chapter, the roles of HLGAGs in tumor progression were further investigated in animal tumor models paralleled with a complete mechanistic study for the regulatory activities of HLGAGs.
Chapter 4

In vivo Roles of HLGAGs in Tumor Progression

Summary

This chapter is a direct extension of the research from the previous chapter. Consistent with the in vitro observations, heparinase III again inhibited primary tumor growth and secondary lung metastasis in vivo, while hep I had either minimal or opposite effect on tumor progression in the same model systems. Further, the regulatory effects of heparinas I and III were found to be mediated through the HLGAG fragments released from the cell surface as a result of enzymatic degradation. These fragments showed distinct, often opposite, effect on tumor progression. Immunocytochemistry study of tumor tissues revealed that both tumor and endothelial cell compartments were affected by heparinase/HLGAG fragment treatment. Additional immunoblotting studies identified specific cell signaling pathways that are affected by HLGAG targeting. FGF2 mediated signaling was shown to be the principal signaling pathway regulated by heparinase treatment of tumor. The research provided the first direct evidence showing HLGAGs being critically involved in process of tumor development, and showed that cell surface HLGAG chains contain “cryptic modulators” of tumor progression. This part of thesis research has resulted in one submitted paper and one patent protection.
4.1 Methods

Effect of heparinase treatment on tumor growth

$4 \times 10^5$ log growth phase B16BL6 melanoma cells in 0.1 ml PBS were injected subcutaneously to the right flank of C57BL/6 mice on day 1 ($n = 5$). Daily injection of 0.1 ml of PBS, heat-inactivated hep III or active hep III started on day 4 and continued throughout the experiment. In addition, osmotic pumps (100 $\mu$l capacity delivering at 0.5 $\mu$l per hour) containing PBS or 3 mg/ml hep III were implanted subcutaneously at a place remote from the tumor site. Mice were sacrificed on day 15. Tumor volume was measured daily after day 7 with a caliper and calculated with the formula [volume ($mm^3$) = $0.52 \times$ (width)$^2 \times$ (length)].

Effect of heparinase treatment on tumor metastasis

Cells in log growth phase were briefly trypsinized and resuspended in PBS to a final concentration of $1 \times 10^6$ per ml. Prior to injection, cells were treated with either PBS, hep I or hep III for 30 min at $37^\circ$C. 0.2 ml cell suspensions ($2 \times 10^5$ cells) were injected slowly via tail vein ($n = 8$). Mice were sacrificed on day 14 and lungs were harvested and rinsed with water. The number of nodules on the lung surface was counted with the assistance of a dissection microscope.

Preparation of HLGAG fragments by heparinase treatment of tumor cells

HLGAG fragments were prepared as follows: 90-100% confluent B16BL6 cells were washed with PBS twice. 1.5 ml of PBS containing 200 nM of hep I or hep III were added to the flasks, incubated at $37^\circ$C on a shaker for 1 h. Supernatant was pooled into a tube, centrifuged for 8 minutes at 4500 x g, boiled for 15 minutes and filtered. The concentration of HLGAG fragments was determined by capillary electrophoresis by comparing to standards.

Effect of tumor cell derived HLGAG fragments on tumor growth

$4 \times 10^5$ B16BL6 cells were injected subcutaneously as described on day 1. Osmotic pumps (200 $\mu$l capacity delivering 0.5 $\mu$l per hour) were implanted subcutaneously on day 2. Daily subcutaneous injection of 0.1 ml HLGAG fragment solution and PBS was started on day 5 and continued throughout the experiment ($n=5$ or 6). The mice were euthanized on day 15.
Effect of tumor cell derived HLGAG fragments on tumor metastasis

2 x 10^5 of B16BL6 cells resuspended in PBS, hep III generated B16BL6 saccharide fragments and hep I generated B16BL6 saccharide fragments solutions were injected via tail vein of mice (n = 7 or 8) and the lungs were harvested and analyzed as described.

Cell kinetics study of primary tumor

Immunohistochemistry was performed on tumor sections in the following manner. Briefly, tumor tissues were fixed in either 4% (vol/vol) formaldehyde overnight for von Willebrand factor (vWF) staining and terminal deoxynucleotide transferase (TdT) labeling, or in Glyo-Fixx solution overnight for Ki-67 nuclear antigen staining. Tissues were embedded in paraffin according to standard histological procedures. For vWF staining, sections (5 μm thick) were incubated with 0.2 N HCl for 10 min., autoclaved in a Coplin jar immersed with target retrieval solution (Dako) for 15 min., and permeabilized with 2 μg/ml proteinase K at 37°C for 15 min. Sections were incubated with rabbit anti-human vWF antibody coupled with horseradish peroxidase (HRP) (Dako). Positive staining was detected by substrate reaction with diaminobenzidine. Sections were counterstained with Gill’s hematoxylin and mounted with Permount (Fisher). Ki-67 antigen staining (rabbit anti-human Ki-67 antigen antibody coupled with HRP, Dako) and TdT labeling (DeadEnd Colorimetric Apoptosis Detection System, Promega) were done essentially according to manufacture’s protocol. Capillary density was determined by counting the number of vWF-positive capillaries per high power field (HPF, x200). The proliferative and apoptotic indices of tumor cells within areas of viable tumor were estimated from the percentage of cells scored under a light microscope at 400-fold magnification. A minimum of 2000 cells was counted in each animal.

Construction of 2-OST antisense construct and transfection of B16 melanoma cells

Heparan sulfate 2-O sulfotransferase (2OST) gene in pBluescript vector was used as the template for antisense construction. Primers spanning the 5’ untranslated region and the transmembrane domain of 2OST were used to obtain PCR product of 209 bp oligonucleotide sequence. Xho I and Bam H1 restriction sites were engineered into the 5’ and 3’ of the target sequence via PCR procedure. The primer sequences are:
CTCGAGCTTGATCTCCAGCCGCCGTTTCATGGGGCTCCTCAGGATCATGATG (5’ primer) and GGATCCAGGGCATCCATTGTATGCCGCTG (3’ primer). The PCR product was subcloned into the vector pCR2.1 (in vitrogen). The insert in pCR2.1 vector was cut out with Xho I and Bam H1 and cloned into pCDNA3.1 vector in antisense orientation. This antisense vector was used to transfect B16BL6 melanoma cells and stably transfected cells were selected with G418 at 800 mg/ml in αMEM. The stable transfected B16 cells were then used for tumor lung metastasis study as described for B16 cells.

Compositional analysis of HLGAG fragments

Saccharide fragments were collected in PBS, and subjected to purification and fractionation. First, samples were bound to an Ultrafree-DEAE membrane, which had been equilibrated with pH 6.0 sodium phosphate, 0.15 M NaCl. They were washed with the same buffer and eluted with 0.1 M sodium phosphate buffer pH 6.0 that contained 1.0 M NaCl. The fragments were then concentrated and buffer exchanged into ultrapure water by application to a microcon column (MWCO= 3,000 Da). The sample was digested overnight with a cocktail of hep I-III (1 mU each) in 25 mM sodium acetate 1 mM calcium acetate, pH 7.0. Analysis was completed by capillary electrophoresis using a high sensitivity flow cell under reverse polarity with a running buffer of 50 mM tris/ phosphate pH 2.5. Disaccharide identification was made by comigration with known standards. For mass spectrometric oligosaccharide mapping of HLGAG saccharide fragments, hep I or hep III-derived HLGAG saccharide fragments were subjected to partial enzymatic cleavage by 100 nM (8 µg/ml) heparinase II in 10 mM ethylenediamine, 10 µM ovalbumin, 1 µM dextran sulfate pH 7.0 for one hour. Resulting digests were complexed with the basic peptide (RG)19R and subjected to matrix-assisted laser desorption ionization mass spectrometry.

Effect of treatment of tumor with hep I and hep III in FGFR1 activation compared to PBS

Level of phosphorylated FGFR1 in tumor samples was assessed by standard immunoprecipitation followed by Western blotting with phosphotyrosine specific antibody. Primary B16BL6 tumors were grown and treated as described earlier and at day 15 the tumor was harvested in cold modified RIPA buffer containing enzyme inhibitors and homogenized. The homogenates were past through 25-gauge needle 3 times and centrifuged. The supernatant
was adjusted for protein concentration using the Bio-Rad protein assay kit (Bio-Rad). FGFR1 was immunoprecipitated with poly-clonal anti-FGFR1 antibody (Santa Cruz Biotechnology, Inc., CA). Samples were then pelleted, washed, and eluted from the beads by addition of sample buffer and boiled for 3 minutes. After electrophoresis, the gel was transferred to nitrocellulose membrane by standard methods. The immunoblot was probed with phosphotyrosine specific antibody conjugated to HRP (RC20; Transduction Laboratories, Lexington, KY) and developed with SuperSignal West Pico Chemiluminescent substrate. The molecular weight of FGFR1 is 120 KDa.

Effect of heparinase treatment on activation of FAK in B16BL6 tumor

The tumor samples were prepared for western blotting as described above. The FAK protein was immunoprecipitated with mouse anti-FAK monoclonal antibody (Transduction Laboratories, Lexington, KY) according to the procedures described above. The phosphorylated FAK was detected using phosphotyrosine specific antibody RC20.

Level of total and phosphorylated Erk-1, 2 in heparinase-treated B16BL6 tumor

Tumor homogenates were prepared and processed as described. The supernatant was used for total protein concentration assay and immunoblotting. The immunoblot was detected with anti-phospho-Erk-1, 2 or anti-Erk-1, 2 as described.

Effect of heparinase treatment on Akt activation

The primary tumor was treated and processed as described above. Akt antibody and phospho-Akt antibody from New England Biolabs were used to probe the immunoblot. The detection was done as described.

FGF-mediated proliferation of BaF3 cells with transfected FGFR in the presence of HLGAG fragments generated with either hep I or III

BaF3 cells expressing FGFR were grown in the following fashion. The initial cell number was counted by Coulter counter, and resuspended to a density of $1 \times 10^5$ cells/ml into 12 samples of 6 ml. Each sample of cells was centrifuged 3 min at room temperature at 1085 x g, and resuspended in HLGAG preparations in PBS, producing two sets of cells in the same media.
One of each set was supplemented with 50 ng/ml FGF2 while the other was unsupplemented. 1 ml from each set was added to each of 3 wells on a 24-well tissue culture plate. The cells were incubated for 72 hr at 37 °C/5% CO₂. Whole cell number was counted at the experimental endpoint by Coulter Counter. This procedure was repeated three times. Collected data was normalized using a proliferative index (PI), as previously described. The index is defined as the increase in cell number for the experimental case divided by the increase in cell number for the positive control. The positive control was cells in DMEM with 10% BCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 500 ng/ml heparin, and 50 ng/ml FGF2. The negative control was cells in DMEM with 10% BCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 500 ng/ml heparin

Modulation of FGF2 activity in vivo by B16BL6 fragments

Hydron pellets containing FGF2, hep I fragments with FGF2, and hep III fragments with FGF2 were prepared as described. The amount of FGF2 loaded into each pellet was ~120 ng, and the amount of HLGAG fragments was approximately 0.2-2 ng. Sprague-Dawley rats (male, 350-400 g) were anesthetized with Ketamine (80 mg/kg) and Xylazine (10 mg/kg). An incision is made in parallel to the limbus at 2 mm distance, and a pocket is made with a #11 surgical blade. On day 6 after the implantation into the cornea of Sprague-Dawley rats (n=5), the corneal neovascularization was photographed with a slit lamp and the extent of neovascularization was expressed as linear length and circumferential clock hours as described.

4.2 Results and Discussions

Substrate specificity of the heparinases dictates whether they promote or inhibit tumor growth and metastasis.

Heparinases I (hep I) and III (hep III), which have very distinct HLGAG substrate specificities, were employed as tools to investigate the role of HLGAGs in tumor growth, neovascularization and metastasis. Hep I cleaves at the highly sulfated regions of HLGAGs, while hep III only cleaves at the undersulfated regions of the polysaccharide chain (Figure 1.1). Since these enzymes cleave divergent regions of HLGAGs, leaving behind intact structurally distinct saccharide fragments, they have become powerful tools to investigate the in vivo and in vitro roles of HLGAGs in processes such as development and neovascularization. B16BL6
melanoma was used as a model system and tumor-bearing mice were treated with either hep I or III to investigate their effect on tumor progression (Figure 4.1). Hep I delivery, through controlled release via an osmotic pump, accelerated tumor growth in treated mice (Figure 4.1B). This is consistent with the current model of heparanase expression being associated with tumor progression. Conversely, delivery of hep III dramatically inhibited primary tumor growth in a dose dependent fashion (Figure 4.1A, B). The opposing effects on tumor

![Graphs showing tumor volume and number of lung nodules](image)

**Figure 4.1.** Effect of heparinase treatment on tumor growth and metastasis. A. PBS (left), inactive heparinase III (hep III) (center), and active hep III (right) treated mice with B16BL6 melanoma 15 days after tumor implantation. B. The growth curves of mice bearing melanoma treated with PBS, hep I, inactive hep III and active hep III. C. Lung metastasis of B16BL6 melanoma 13 days after tail vein injection of B16BL6 cells. * Indicates p < 0.05 (Mann-Whitney test) and error bars represent standard error (SE). It should be pointed out that mice were systemically examined at the time of necropsy and no signs of pathological changes or inflammatory responses were found at non-tumor bearing organs or tissues.

The growth observed by these two enzymes suggest that differences in their substrate specificity play a specific role in modulating tumor growth and that in vivo HLGAGs are more than a passive barrier to tumor metastasis. To explore further hep III’s anti-tumor activity, more extensive
studies were completed to understand how the action of hep III inhibits tumor growth, using hep I treatment to provide a frame of reference for these studies.

Figure 4.2. Inhibition of primary tumor growth by hep III treatment. A. The tumor was grown and treated as described in methods. In this experiment, mice were treated with daily injections of hep III (2 mg/ml) every 12 h without pump implantation. As a result the dose received is 20 mg/kg/day. B. Inhibition of B16BL6 tumor growth by hep III in nude mice. Again, B16BL6 tumors were grown and treated as described in methods. Starting on day 4 after tumor cell injection, hep III (2 mg/kg) was injected subcutaneously daily to the treatment group throughout the experiment at a dose of 10 mg/kg/day while the control group received a PBS injection only. Mice were sacrificed on day 15. Tumor volume was measured and calculated as described.

At a dosage of 12 mg/kg per day of hep III, about 75% inhibition of tumor growth was observed. The route of administration was found not to play a significant role in heparinases' activity as repeated subcutaneous or IP injections had a similar effect as delivery by osmotic pump (Figure 4.2A). Specifically, in a separate experiment, B16 melanoma bearing mice were treated with either s.c. or i.p. injection of heparinase III. The inhibition of the primary tumor growth was found to be comparable to the mice treated with controlled release via osmotic pump. Control mice treated with heat-inactivated hep III exhibited comparable growth curves to those of mice treated with PBS, indicating that the catalytic activity of hep III alone was responsible for its ability to inhibit primary tumor growth. (Figure 4.1A, B). In addition, hep III inhibited B16BL6 growth in nude mice at a level comparable to that seen in immunocompetent mice (Figure 4.2B), suggesting the inhibition was not immune cell-mediated. To ensure that these observations were not limited to the tumor model chosen, hep III was used to treat mice bearing Lewis lung carcinoma (LLC) tumors (Figure 4.3A). Similar to what was observed in the
B16BL6 tumor model, hep III at a dose of 12 mg/kg per day showed marked inhibition of tumor growth. Thus, the in vivo studies, along with in vitro cell culture experiments points to the enzymatic action of hep III reducing the tumorigenicity of a variety of tumor cell types.

**Figure 4.3.** A. Growth curves of primary tumor growth for LLC tumor in C57BL/6 mice treated with either PBS or heparinase III. $4 \times 10^3$ log phase LLC cells were injected to the flank of mice on day 1. Daily injection of 0.1 ml of either PBS or 3 mg/ml recombinantly expressed heparinase III started at the day 4 and continued throughout the experiment. At day 8, osmotic pumps (100 µl capacity delivering at 0.5 µl per hour) containing PBS or 3 mg/ml hep III were implanted subcutaneously at a place remote from the tumor site. Mice were sacrificed at day 20. B. Lung metastasis of LLC cells injected through tail vein. Log growth LLC cells were trypsinized for 30 seconds and resuspended in PBS to a final concentration of $2 \times 10^6$ per ml. For experimental group, cells were incubated with 200 nm hep III for 30 minutes at 37°C before injection via tail vein. Mice were euthanized 12 days after tail vein injections, lungs were harvested, rinsed in tap water and fixed overnight in Bouin’s solution. The number of nodules on lung surface were counted with the aid of a dissection microscope. * Indicates p < 0.05 (t-test).

Heparinase treatment of tumor cells modulates tumor metastasis.

To investigate the role of HLGAGs in tumor metastasis, B16BL6 cells were treated with either hep I or III, injected into the tail vein of syngeneic mice, and assessed for their ability to colonize to the lungs. While the metastatic potential of hep I-treated cells was comparable to that of PBS-treated cells, hep III-treated B16BL6 cells were significantly less able to colonize to the lungs (Figure 4.1C). Similarly, treatment of HLGAG coat on the surface of LLC cells by hep III inhibited their ability to metastasize and colonize the lungs (Figure 4.3B).
Figure 4.4. Effect of tumor cell derived HLGAG fragments on (A) tumor growth and (B) metastasis. A. Primary tumor growth of B16BL6 melanoma. Shown in A are growth curves of mice treated with hep I (2 μg/kg/day), hep III (10 μg/kg/day) derived B16BL6 saccharide fragments, and PBS control. B. Lung metastasis of B16BL6 melanoma. 2 x 10^6 of B16BL6 cells resuspended in PBS (far left), or in hep III generated B16BL6 saccharide fragments (2nd from left) and in hep I generated B16BL6 saccharide fragments (2nd from right) solutions were injected via tail vein of mice (n = 7 or 8) and the lungs were harvested and analyzed as described. Shown on top of B are representative lung metastases from PBS and hep III fragments-treated groups. Also shown in the figure is the effect of the stable expression of a 2-O sulfotransferase antisense [2OST(-)] 226 construct on the metastatic potential of B16BL6 cells (far right). In each case, effects of the saccharide fragments were not altered upon chondroitinase ABC treatment (data not shown). It should also be pointed out that there were no signs of morbidity such as weight loss associated with any of the treatments. * Indicates p < 0.05 (Mann-Whitney test). Error bars represent SE.

There are two possible explanations for the opposing effects of hep I and hep III treatment on tumor growth and metastasis. First, it is possible that heparinase treatment directly impinges on the growth of the tumor. If this is the case, then heparinase treatment digests the HLGAG coat on the tumor cell surface, thus altering the growth and metastatic ability of the tumor cells. Alternatively, heparinase treatment might indirectly influence the growth and metastasis of the tumor through the release of bioactive HLGAG fragments from the cell surface. In this case, it is these bioactive fragments themselves, and not the enzyme per se, that modulate
tumor growth and metastasis. If this is the case, then treatment of B16BL6 cells with either hep I or hep III, with differing substrate specificity, release HLGAG fragments of distinct composition that have opposite effects on the tumorigenicity of B16BL6 cells. In support of this second explanation, ex vivo digestion of the HLGAG coat present on tumor cells with either heparinase, followed by centrifugation and resuspension in PBS to remove the enzyme and HLGAG fragments released from the cell surface, prior to in vivo injection results in the cells being functionally identical to controls. Thus, the released tumor cell HLGAG fragments appear to play the key role in modulating tumor growth and metastasis.

**Antisense blocking sulfation of HLGAGs regulate tumor metastasis**

The results presented above suggest that cell surface HLGAGs regulate tumor metastasis in a sequence-dependent manner. To directly test whether this speculation is factual, antisense constructs targeting specific HLGAG synthesis enzymes were used to transfet tumor cells. Stably transfected tumor cells were tested for their ability to colonize the lung after tail vein injection. The success of antisense blocking of 2-OST was confirmed by compositional analysis of HLGAGs at the cell surface of 2-OST (-) transfected B16 cells, which indicated that they were primarily undersulfated with a clear deficiency in 2-O sulfation. Of note is the fact that the HLGAG composition of these mutants is more similar to the composition of HLGAGs derived from hep I-treated B16BL6 cells than that of the HLGAGs derived from hep III-treated B16BL6 cells. Consistent with the increase in tumorigenicity of hep I-treated B16BL6 cells, B16BL6 cells expressing the 2-O sulfotransferase antisense construct had a dramatically increased ability to metastasize (Figure 4.4B).

**Tumor cell HLGAG fragments modulate tumor growth and metastasis**

To investigate directly the role of HLGAG fragments in modulating tumor progression, hep I and hep III-generated tumor cell HLGAG fragments were isolated, harvested in PBS and their in vivo activity tested. Treatment of mice with HLGAG fragments generated by hep I treatment promoted primary tumor growth (Figure 4.4A), consistent with hep I’s enzymatic effect as noted in Figure 4.1. On the other hand, hep III-generated HLGAG fragments showed significant inhibition of primary tumor growth by about 70% (Figure 4.4A), which is comparable with hep III’s enzymatic effect on primary tumor growth as noted in Figure 4.1.
Notably, the pronounced biological potency of both hep I and hep III treatment could be recapitulated with injection of the fragments alone, suggesting that the effects of enzyme treatment are due entirely to the release of bioactive HLGAG fragments from the cell surface of B16BL6 cells. To confirm that this is the case, when B16BL6 cells were suspended or ‘doped’ in a PBS solution containing hep III-generated B16BL6 HLGAG fragments at very low concentration (2.0 μg/ml), prior to injection via tail vein of mice, it was found that these cells were 75% less able to form lung metastases, suggesting that the HLGAG fragments were extremely potent inhibitors of tumor metastasis (Figure 4.4B). Taken together, these results are consistent with the following model. Hep I treatment releases bioactive HLGAG fragments from the surface of the tumor cell that are able to promote primary tumor growth. Hep III, with an orthogonal substrate specificity, releases HLGAG fragments from the tumor cell surface that inhibit both primary tumor growth and tumor metastasis.

HLGAG fragments with distinct composition are potent inhibitors of tumor growth and metastasis.

Compositional studies of the HLGAG saccharide fragments generated upon heparinase treatment confirmed that the HLGAG fragments released from B16BL6 cells by hep I or hep III are compositionally different and structurally distinct (Figure 4.5A-F). Capillary electrophoresis, in combination with exhaustive enzymatic digest, was used to derive compositional information on the saccharide fragments (Figure 4.5)\textsuperscript{204}. The saccharide fragments derived from hep III treatment had more of tri and di- sulfated disaccharides while the hep I-treated HLGAGs had more mono and un-sulfated disaccharides (Figure 4.5A, B). This is consistent with the known substrate specificities of the heparinases. In addition, mass spectrometric investigation of HLGAGs \textsuperscript{204}, yielded a “fingerprint” of oligosaccharides generated from each of the treatments and proved that the saccharide fragments generated from the different treatments are structurally distinct (Figure 4.5E, F).
Figure 4.5. Compositional analysis of HLGAG saccharide fragments released from B16BL6 cells. B16BL6 cells were treated with either A, hep I; B, hep III; or C, a PBS control and the released HLGAG fragments harvested. Disaccharide identification was made by comigration with known standards, identity of peaks is enumerated in A and B. D, Table showing the relative percentage of the HLGAG disaccharides in hep I and hep III-generated fragments. The percentage was obtained based on the normalized peak areas of the different disaccharides in A and B. Note that the relative composition of the hep I and hep III-generated fragments are very different. The alphanumeric assignment of each disaccharide is also listed as outlined previously. Saccharide analysis of the B16BL6 cells that were transfected with the 2OST(-) indicated that there was an absence of 2-O sulfate-containing saccharides, specifically the trisulfated disaccharide ΔU₃g-HNS₆S (data not shown). Mass spectrometric oligosaccharide mapping of hep I (E) and hep III (F) derived HLGAG saccharide fragments. The HLGAG fragment fingerprint is different for the hep I vs hep III generated fragments consistent with each being structurally distinct.

HLGAG fragments target both the tumor cell and endothelial cell compartments.

To understand how hep I, hep III or heparinase-generated HLGAG fragments elicited their function, histological and cell kinetic studies were completed (Table 4.1). For hep III treated tumors, immunohistological examination of tumor samples revealed reduced
neovascularization\textsuperscript{121}, increased cellular apoptosis and decreased cellular proliferation compared to that of PBS-treated animals (Table 4.1). Similar results were observed upon immunohistological examination of animals treated with hep III-generated HLGAG fragments, consistent with the notion that bioactive HLGAG fragments are indeed the mediators of tumor growth and neovascularization. Immunohistological examination of animals treated with hep I-generated HLGAG fragments revealed the opposite, namely increased proliferation, decreased apoptosis, and increased neovascularization (Table 4.1). Surprisingly, it appears as if the release of bioactive HLGAG fragments through heparinase treatment affects tumor physiology both by modulating the survival and proliferation of the tumor cell compartment as well as influencing the ability of the endothelial cell compartment to form new blood vessels.

**Mechanism of action:** HLGAGs impinge on the biological activity of specific signaling molecules.

Having observed the marked and opposite effects that distinct HLGAG fragments have on both the tumor and vascular compartments, the underlying molecular mechanism of HLGAGs in tumor progression was further investigated. As many HLGAG binding proteins are growth factors and cytokines, HLGAG-binding growth factors that play key roles in tumor pathobiology were explored to identify an immediate target of the HLGAG fragments generated from the surface of tumor cells. FGF2 signaling has been shown to be a prerequisite for melanoma progression promoting tumor growth in an autocrine fashion, and the interruption of the FGF2 autocrine loop by interfering with either FGF2 or FGF receptor (FGFR) activity results in inhibition of melanoma progression\textsuperscript{124,127,128}. On the other hand, upregulation of the expression of FGF2 in normal melanocytes result in their malignant transformation\textsuperscript{124}. Furthermore, FGF2 is a potent and essential angiogenic factor regulating melanoma neovascularization\textsuperscript{126,130}. Most importantly, specific HLGAG structures are known to bind and modulate FGF2 activity, and there is increasing evidence that, HLGAG sequences, depending on their structure, can either promote or inhibit FGF2 activity\textsuperscript{6,70,131,233}. Given the multiple lines of evidence implicating FGF2 as a key switch in melanoma progression, taken together with FGF’s strict dependence on
Table 4.1. The results of immunohistochemical analysis of tumor samples. # Mean and SE.

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<td>15.1 ± 0.75</td>
<td>23.3 ± 0.71 *</td>
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* indicates P < 0.05 (Mann-Whitney test).

Figure 4.6. FGF2 signaling is modulated by HLGAG fragments. A. FGF-mediated proliferation of BaF3 cells with transfected FGFR in the presence of HLGAG fragments generated with either hep I or III. Collected data was normalized using a proliferative index (PI), as previously described. The index is defined as the increase in cell number for the experimental case divided by the increase in cell number for the positive control. B. Effect of treatment of tumor with hep I and hep III in FGFR1 activation compared to PBS. C. Effect of heparinase treatment on activation of FAK in B16BL6 tumor. D. Level of total and phosphorylated Erk-1, 2 in heparinase-treated B16BL6 tumor. E. Effect of heparinase treatment on Akt activation. The primary tumor was treated and processed as described above. Akt antibody (upper panel) and phospho-Akt antibody (lower panel) from New England Biolabs were used to probe the immunoblot.
HLGAGs for its activity $^{70,131,233}$, further researches were conducted to determine whether the immediate target of tumor-derived HLGAG fragments is indeed FGF2.

To test whether hep I and hep III-derived fragments bind to FGF2 and affect its activity, F32 cells, a pre-lymphocyte cell line that has been transfected with FGFR was used. F32 cells often has been used as a model system to study FGF-mediated signaling in cell culture unfettered by complications associated with signaling events initiated by other growth factors and/or receptors $^{234}$. Similar to what was observed in B16BL6 cell culture studies, hep I fragments promote, whereas hep III fragments inhibited FGF2-mediated cellular proliferation in these cells (Figure 4.6A). Together, these in vitro findings point to the fact that HLGAG fragments derived from the cell surface can substantially and specifically affect FGF2 signaling in B16 melanoma.

Consistent with the in vitro observations, it is further realized that hep I and hep III-derived B16BL6 fragments significantly affect FGF signaling pathways in vivo. Within the tumor in the animals, both FGFR phosphorylation in hep I and hep III-treated animals as well as Erk-1 and 2 signaling were examined. Treatment of the primary tumor with hep III (or its generated fragments) inhibited phosphorylation of FGFR1 while hep I treatment had the opposite effect on the phosphorylation of FGFR1 (Figure 4.6B). Consistently, treatment of the primary tumor with hep III resulted in a lower level of activated Erk-1, 2 (Figure 4.6D). Additional intracellular signaling events such as focal adhesion kinase (FAK) activity, which is implicated in cell adhesion and migration processes $^{235,236}$, was similarly modulated by hep I and III treatment of the tumor. Consistent with these findings, hep III treatment inhibited FAK activation (Figure 4.6C). Notably there was no change in activation of Akt with either hep I or hep III treatment (Figure 4.6E), indicating that the changes in phosphorylation were specific and resulted from down-regulation of only certain signaling pathways. Together, these results suggest that HLGAG fragments mediate FGF2 signaling with hep I-derived fragments promoting FGF2 activity and hep III-generated fragments inhibiting it. This effect was observed in key steps of FGF-mediated signaling, from the cell surface receptor (FGFR) through downstream signaling events.

To demonstrate a direct interaction between B16BL6 HLGAG fragments and their immediate target FGF2 in vivo, the ability of B16BL6 HLGAG fragments to modulate FGF2-induced responses leading to cell migration, proliferation, and differentiation was evaluated in vivo using corneal neovascularization assay (Figure 4.7; Table 4.2). In this model, hep I-
generated fragments mixed with FGF2 bound to the growth factor and promoted the in vivo neovascularization response to FGF2 (Figure 4.7B; Table 4.2) whereas hep III-generated fragments, mixed with FGF2, bound to the growth factor but dramatically inhibited its activity (Figure 4.7C; Table 4.2). In addition, similar results were observed when HLGAG fragments were formulated as a separate pellet, and placed in between the FGF2 pellet and the limbus.

Table 4.2. The extent of angiogenesis as evaluated by clock hours and vessel length \(^a\).

<table>
<thead>
<tr>
<th>Response</th>
<th>FGF2</th>
<th>FGF2 + Hep I frag.</th>
<th>FGF2 + Hep III frag.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clock Hours</td>
<td>2.4 ± 0.24#</td>
<td>3.4 ± 0.19 *</td>
<td>0.2 ± 0.12 *</td>
</tr>
<tr>
<td>Vessel Length (mm)</td>
<td>1.9 ± 0.05</td>
<td>2.0 ± 0.07</td>
<td>0.4 ± 0.22 *</td>
</tr>
</tbody>
</table>

\(^a\) According to Kenyon et al. \(^{228}\). \# mean and SE. * P < 0.05 (Mann-Whitney test).

Figure 4.7. Modulation of FGF2 activity in vivo by B16BL6 fragments. A-C, Assessment of FGF2 signaling in vivo with the rat corneal pocket assay. Representative slit lamp photographs of rat corneas on day 6 after implantation with Hydon pellets (arrow heads) containing (A) FGF2, (B) hep I fragments with FGF2, and (C) hep III fragments with FGF2. The amount of FGF2 loaded into each pellet was \(\sim 120\) ng, and the amount of HLGAG fragments was approximately 1 ng.

The inhibition of neovasularization by hep III generated fragments was found to be dose dependent with initial inhibition observed at about 0.2 ng. This result is consistent with the changes in neovascularization observed in the immunohistological study of tumor. Taken together, the above results indicate that a direct in vivo target of the HLGAG fragments released by heparinase treatment is FGF2. Based on the current understanding of HLGAG-FGF interactions \(^6,106,131\), it can be reasoned that hep I-generated fragments act to bridge FGF2 to its
cognate receptor activating intracellular signaling pathways, while hep III-derived fragments are antagonists, preventing the formation of a signaling complex at the cell surface. Thus, by either directly activating or inhibiting FGF2 signaling, these bioactive fragments are potent modulators of tumor growth and metastasis. The results presented here do not preclude a direct or indirect effect of HLGAG fragments on other HLGAG binding growth factors playing a role in tumor pathophysiology. However, based on the many lines of evidence presented here, it appears that FGF2 is indeed an immediate target for enzymatically derived HLGAG fragments.

4.3 Implications

The results presented herein demonstrate that by impinging on the biological activity of specific signaling molecules, HLGAGs play a direct role in tumor growth and metastasis. Most importantly, HLGAGs at the cell surface of tumor cells contain both ‘activatory’ and ‘inhibitory’ HLGAG sequences that are in balance. The specific degradation of one set of sequences (e.g., by hep I) results in the release of fragments that promote the biological activity of HLGAG-binding signaling molecules, and thus act as a switch for tumor growth and metastasis. Conversely, degradation by an enzyme with an orthogonal substrate specificity (e.g., hep III) tips the balance in the opposite direction, releasing fragments that antagonize HLGAG-binding signaling molecules, leading to the inhibition of tumor growth and metastasis. Thus, it was demonstrated here for the first time that chemically complex HLGAGs at the cell surface are “cryptic” promoters or inhibitors of tumor growth and metastasis that become biologically active upon their release from the cell surface by specific HLGAG degrading enzymes.

Just as collagenases clip the proteinaceous compartment of the ECM, serving either to increase tumor growth (e.g., breakdown of the basement membrane) or to inhibit tumors (e.g., the formation of endostatin from collagen XVIII), the polysaccharide compartment exhibits a similar phenomenon. Importantly, like the proteolytically cleaved collagen fragment endostatin, distinct HLGAG oligosaccharides upon release by enzymatic cleavage from the tumor cell surface can serve as potent inhibitors of tumor progression. Thus, the present study not only allows a new paradigm of how the polysaccharides modulate tumor growth and metastasis, but it identifies a novel therapeutic target by providing a framework towards the development of HLGAG-based novel anti-cancer molecules.
PART IV
PULMONARY DELIVERY OF HLGAG-BASED THERAPEUTICS

Chapter 5
Pulmonary Delivery of HLGAGs as Dry Aerosol Powder

Summary

HLGAGs are becoming increasingly important in medicine for their diverse biological activities including anticoagulation, antithrombosis, anti-clastase, anti-tumor and anti-inflammatory effects\textsuperscript{8,143,239,240}. Due to their rapid metabolism after oral administration, the clinical use of HLGAGs, such as heparin, has been limited to injection, which is inflicted with side effects at the injection site and poor patient compliance. Lung has the largest absorption mucosa area in human body and is regarded as an ideal drug delivery site. Past attempts in delivering HLGAGs by pulmonary inhalation of liquid aerosol was met with failure\textsuperscript{241-243}. This chapter of thesis research presented the results of delivering HLGAGs to the lung as dry aerosol particles. Dry aerosolized HLGAGs of various particle properties were shown to be able to deliver HLGAGs to the lungs and be absorbed efficiently. Extensive pharmacokinetics studies on the effect of formulation and particle physical properties on delivery efficiency were completed. The mechanism of absorption for inhaled HLGAGs was investigated. Further, pulmonary inhalation of HLGAGs was shown here to be safe and without significant pulmonary absorption resistance. These results have resulted in one patent protection and 2 papers under preparation.
5.1 Motivation and Background

In the previous chapters, this thesis research demonstrated the potential clinical application for HLGAGs in cancer therapy. In addition to this, HLGAGs, such as unfractionated heparin (UFH) and low molecular weight heparin (LMWH) have been widely used in clinic as anticoagulant and antithrombotic agents for decades \(^{159,239}\).

The clinical uses of anticoagulant and antithrombotic effect of HLGAGs can be roughly classified as treatment and prevention \(^{244-246}\). HLGAGs (UFH and LMWHs) are the standard choice for prophylaxis of thrombosis following orthopedic surgeries, prosthesis replacement surgeries and other surgeries where prolonged immobilization of the patients is required. Equally importantly, heparin has been widely used in prevention and treatment of thrombosis in cardiovascular diseases such as acute myocardial infarction, unstable angina, ischemic stroke, atrial fibrillation and etc. Heparin has been used in the treatment of deep venous thrombosis (DVT), pulmonary embolism (PE) and other vascular conditions \(^{245,247-249}\).

The rapid metabolism in gastro-intestinal tract prohibited the oral administration heparin, and parenteral injection (intravenous and subcutaneous) has been the only administration choice. Since subcutaneous (s.c.) injection of UFH results in poor pharmacokinetics, UFH is administered by intravenous (i.v.) injection. Due to the potentially dangerous side effects of UFH, i.v. administration of UFH is increasingly being replaced by s.c. administration of LMWHs. As a result, s.c. administration of LMWHs is becoming increasingly important in prevention of thrombotic diseases. Although s.c. administration of LMWHs is associated with improved bioavailability and less interindividual variation, twice daily injections are still not the most convenient for long term therapy since repeated s.c. injections are known to cause irritation and ulceration at the site of injection. As a result, the use of parenteral heparin is typically restricted to the hospital setting. Under current practice, a course of hospital treatment with heparin is routinely followed by a 20 to 90 day or longer course of out-patient treatment using warfarin, the only oral anti-coagulant widely available. However, warfarin has a slow onset of action with long half-life and a significant number of drug interactions with FDA approved medications \(^{176}\).

For this reason, to this date, much effort has been devoted to develop alternative administration routes to the existing injection method without clear success. Among them,
intrapulmonary delivery of heparin has attracted much attention since alveoli contain the largest absorption surface in the human body. Thin barrier between the rich capillary bed and the alveolar space coupled with the very high blood flow rate makes alveoli of lungs one of the most desirable drug delivery sites\textsuperscript{250}. Delivering heparin by conventional liquid aerosol spray or instillation only received limited success due to its poor penetration to the deep lung and resultant poor and unpredictable pharmacokinetics\textsuperscript{251,252}. Less than 10% of the delivered dose as liquid heparin was shown to reach the deep lung and blood circulation\textsuperscript{241,250,251,253,254} and no heparin is detectable in blood when the doses equivalent to therapeutic s.c. or i.v. administration are used\textsuperscript{241,252-254}. Exceedingly high dose of heparin is required to achieve even moderate heparin level in the blood circulation. Additionally, the absorption of heparin after pulmonary inhalation or instillation of liquid heparin is slow and erratic. Other routes of administration of heparin have also failed. These other routes include sublingual, rectal, buccal and transdermal delivery\textsuperscript{252}.

The current knowledge on intrapulmonary delivery of heparin and low molecular weight heparin can be summarized as below.

1. Whether given as liquid aerosol or liquid instillation, the bioavailability of intrapulmonary delivery of heparin or LMWH is consistently less than 10% that of s.c. or i.v. administration. When delivered as liquid heparin aerosol, only less than 10% of delivered dose can potentially reach the deep lung with majority trapped in the upper airways.

2. When given the same doses as what is being used for s.c. or i.v. administration, no detectable heparin is found in the blood circulation after pulmonary delivery. 8-10 times higher dose of heparin is required for intrapulmonary heparin to obtain comparable heparin concentration in the blood circulation to that of s.c. administration.

3. The absorption of heparin from lung after intrapulmonary delivery of liquid heparin is much slower than that of s.c. administration. $t_{\text{max}}$ (the time when peak activity is reached) is typically observed 5 hours after intrapulmonary delivery. Substantial amount of heparin remained in the lung many hours after inhalation.

4. There is no close dose-response relationship observed after intrapulmonary delivery of heparin, a considerable variation in the pharmacokinetics of intrapulmonary heparin is reported.
Thus, other than injection (s.c. and i.v.), none of above mentioned parenteral routes succeeded in efficient delivery of heparin into the circulation. After careful examining the histology of delivery sites for non-injection methods in comparison with s.c. and i.v. injection administration, several common characteristics were found to be shared by these non-injection routes of administration. First, the delivery sites of heparin are relative poor in capillary circulation. For instances, upper respiratory tract epithelial surface, buccal pouches, and rectum. Second, the heparin depot location is separated from capillary circulation by multiple membrane structures, which include all the delivery sites mentioned above except for the s.c. and i.v. injection as well as the deep lung. Finally, these lipid membrane structures are relatively impermeable to hydrophilic macromolecules such as HLGAGs which are highly negatively charged polymers. s.c. administration of heparin allow heparin to directly contact capillary vessels and the limitations listed above are less obvious, which explains the efficient absorption of heparin especially low molecular weight heparin after s.c. injection.

Based on the knowledge drawn above, we realized that, in order to achieve efficient absorption of heparin (UFH and LMWH), it is necessary to deliver heparin to the close proximity of a rich capillary network. Deep lung has the richest capillary network found in an organ in the human body, and the respiratory membrane separate capillary lumen from alveolar air space is very thin (6 μm) and extremely permissive due to the fenestrated membrane structures. In addition, the liquid layer lining the alveolar surface is rich in lung surfactants, which can facilitate the diffusion of HLGAGs across the membrane by disguising the hydrophilic, charged groups of the heparin polymer.

For these reasons, it is hypothesized that one will achieve efficient absorption of HLGAGs if HLGAGs (UFH and LMWH) can be efficiently deposited directly to the deep lung alveolar surface. This hypothesis seems to explain the reported results in intrapulmonary inhalation of liquid heparin aerosol. As reported and mentioned above, only less than 10% liquid aerosol can reach deep lung or lower respiratory tract, but this resulted in a reported approximately 8% bioavailability, meaning most those heparin reached the deep lung got absorbed, which supports our hypothesis.

In general, pulmonary delivery of drugs as dry aerosol particles is known to be more efficient than liquid aerosol in many aspects including better aerosolization, improved loading capacity, more efficient deep lung deposition, and predictable pharmacokinetics. Another
advantage for inhaled drug is that a high therapeutic ratio can be achieved due to the high local concentration in the lung after inhalation, which can be beneficial in treating lung diseases. Therefore, this thesis research set out to investigate the potential for delivery of HLGAGs as dry aerosol particles, using UFH and LMWH as the model drugs.

5.2 Dry Aerosol Preparation and Characterization

Dry aerosol powder preparation

100% HLGAGs (unformulated UFH and LMWH) particles were prepared by using a coffee grinder to a size of 1-500 μm. This powder was then subjected to size separation by sieving through the sieves with mesh sizes of 20, 53, 75 and 106 μm. As a result, powder with sizes ranging from 1-20, 20-53, 53-75, 75-106 and 1-53 μm were obtained. Dry HLGAG particles, with (formulated) or without (unformulated) dipalmitoylphosphatidylcholine (DPPC) as the excipient, were also prepared by using a standard single-step spray-drying process and the particles were analyzed as described. The parameters on the Buchi-191 spray dryer were set as follows: inlet temperature 100-120 °C, liquid flow rate 12 ml/min, aspirator 100%, air flow rate 600-700 L/h. The properties of HLGAG particles are characterized and shown in Table 5.1 and Figure 5.2. In vitro and in vivo activity assays showed no loss of activity of heparin due to the formulation processes.

Dry powder inhalation

In rabbit model, New Zealand male rabbits (2.5-3.0 kg) were used with 3-6 rabbits per group. Rabbits were allowed to adapt for 7 days and free access to water and food. Ketamine (40 mg/kg) and Xylazine (5 mg/kg) were used to anesthetize the rabbits. A-24 gauze Teflon catheter was inserted into the center auricular artery. The catheter was connected to a heparin cap filled with isotonic saline. Then a 15 cm tracheal tube was inserted into the trachea of the anesthetized rabbits via mouth. Two inhalation models were established to evaluate the efficiency of pulmonary delivery of dry aerosolized HLGAGs. In the model 1, the insufflator (Delong Distributors, NJ) attached to a straight delivery tube of equal length to that of tracheal tube was inserted through the tracheal tube (Figure 5.1A). The penetration length was controlled to be about 1 cm above the bifurcation point. The delivery of powder was accomplished by a single puff of 6 ml air through a syringe. Dry aerosolized LMWH powder was delivered at doses of
100, 300, and 600 IU/kg, the amount of powder delivered was determined by subtracting the weight of insufflator at the end of delivery.

In the second rabbit model, a ventilator was connected to the tracheal tube and the ventilator was set to a tidal volume of 120 ml with a respiratory rate of 20 per min (Figure 5.1B). The peak airway pressure was controlled at 15 cm H₂O. The insufflator needle was inserted into a hole drilled at the Y-shaped junction of exhaling and inhaling tube such that the tip of insufflator needle is positioned at the inlet of tracheal tube without penetrating into it. The delivery of powder was accomplished by pushing a syringe attached to the insufflator during the inhaling phase of ventilator-controlled respiration. This model ensures the aerosolized powder particles to travel the whole length of tracheal tube as well as the air-conducting ducts of respiratory system. This method has the potential advantages of 1) simulating that of real pulmonary inhalation seen in human patients; 2) differentiating the powder with better aerodynamic profiles from those with poor aerodynamic performance.

Figure 5.1. Schematic description of the delivery methods in tracheal intubation model (A) and ventilator-assisted inhalation model (B).

0.2 ml of blood was withdrawn 0, 5, 10, 30 min, 1, 2, 3, 4, 6, 8, 10, 12, 14, 18, 24 hours after the inhalation. The first 0.2 ml blood withdrawn was discarded with each withdraw. Blood samples were collected in an aqueous solution of sodium citrate (3.8%; 1/9, v/v), centrifuged at
2000 x g for 20 min and the resulting plasma was shock frozen and stored in −80 °C freezer until assay.

In rat model, male Sprague-Dawley rats weighing 350-450 g (Charles River Laboratories, MA) were housed for 5-7 days prior to experiments. Rats were fed on rat chow, tap water ad libitum. After anesthetization with Ketamine (80 mg/kg) and Xylazine (10 mg/kg), right carotid artery was isolated and intubated with a 22-gauze Teflon catheter. A 3-way stopcock was connected to the catheter for blood sample collection. The blood collection followed the procedures described by Bjornsson and Levy. Pulmonary inhalation was done with the insufflator. The device was weighed prior to and after the inhalation to determine the amount of powder inhaled. The inhalation was accomplished by pushing the plunge of the syringe containing 1.5 ml air two to three times. For each formulation and dose, groups of 5 randomly chosen rats were used.

Subcutaneous administration and instillation of the heparins

In rabbits, HLGAGs (LMWH) at doses of 100, 300 and 600 IU/kg were also given by subcutaneous injection and by i.v. bolus injection via contralateral marginal ear vein. Blood samples were collected 0, 3, 5, 10, 15, 30 min, 1, 2, 3, 4, 6, 8, 12 hours after injection and processed as described above. In rats, 6, 8 mg/kg of UFH and 100, 300, 600 IU/kg of LMWH were also injected by subcutaneous route at an injection volume of 2 ml/kg. LMWH was also instilled through the trachea of the rabbits (n= 3) at 300 and 600 IU/kg in saline (1 ml/kg) via an intubated tracheal tube. The plasma was collected at indicated times and analyzed for anti-Xa activity as described.

Pulmonary lavage study

To determine the rate of disappearance of heparin from the lungs of rabbits after inhalation, lungs were harvested en bloc 0, 5, 30 min, 1, 2, 4, 6, 8 hours after either inhalation or instillation of heparin with two rabbits used for each time point. The trachea was cannulated with an 18 G animal feeding needle and lavaged with five sequential aliquots of 6 ml normal saline. Lavage fluid was centrifuged at 2000 x g for 10 min. The supernatant was shock frozen immediately and transferred to −80 °C until assay. The resulting cell pellets was resuspended in saline, homogenized, and centrifuged. The supernatant was tested for anti-Xa activity. The
lavaged lungs were homogenized in saline (1 g in 5 ml saline) with a polytron device. The
homogenate was centrifuged at 12,000 x g for 10 min and the supernatant was tested for anti-Xa
activity as described below.

**Table 5.1.** The physical properties of dry HLGAG particles.

<table>
<thead>
<tr>
<th>Geometric diameter (µm)</th>
<th>Mass density g/cm³</th>
<th>Aerodynamic diameter (µm)</th>
<th>Porosity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-500, UFH (100%)</td>
<td>0.47 ± 0.1</td>
<td>1 - 350</td>
<td>Nonporous</td>
</tr>
<tr>
<td>1-53, UFH (100%)</td>
<td>0.46 ± 0.1</td>
<td>1- 35</td>
<td>Nonporous</td>
</tr>
<tr>
<td>20-53, UFH (100%)</td>
<td>0.44 ± 0.1</td>
<td>13- 35</td>
<td>Nonporous</td>
</tr>
<tr>
<td>1- 3, UFH (60%) DPPC (40%)</td>
<td>0.3 ± 0.1</td>
<td>0.5- 1.6</td>
<td>Nonporous</td>
</tr>
<tr>
<td>1- 53, LMWH (100%)</td>
<td>0.39 ± 0.1</td>
<td>1- 33</td>
<td>Nonporous</td>
</tr>
<tr>
<td>1-20, LMWH (100%)</td>
<td>0.42 ± 0.1</td>
<td>1- 13</td>
<td>Nonporous</td>
</tr>
<tr>
<td>20- 53, LMWH (100%)</td>
<td>0.43 ± 0.1</td>
<td>13- 35</td>
<td>Nonporous</td>
</tr>
<tr>
<td>53-75, LMWH (100%)</td>
<td>0.45 ± 0.1</td>
<td>35- 50</td>
<td>Nonporous</td>
</tr>
<tr>
<td>75- 106, LMWH (100%)</td>
<td>0.45 ± 0.1</td>
<td>50- 71</td>
<td>Nonporous</td>
</tr>
<tr>
<td>1- 500, LMWH (100%)</td>
<td>0.41 ± 0.1</td>
<td>1- 320</td>
<td>Nonporous</td>
</tr>
<tr>
<td>3-7, LMWH (40%) DPPC (60%)</td>
<td>0.15 ± 0.05</td>
<td>1.2- 2.7</td>
<td>Nonporous</td>
</tr>
<tr>
<td>6- 10, LMWH (40%) DPPC (60%)</td>
<td>0.3 ± 0.06</td>
<td>2- 4</td>
<td>Porous</td>
</tr>
</tbody>
</table>

**Hematology**

To examine the effect of multiple blood withdraws and pulmonary inhalation on the
hematology of animals, blood samples were collected in the beginning and at the end of the
experiment and submitted for analysis. The following parameters were measured: white blood
cell, red blood cell, and platelet counts, hematocrit, hemoglobin. The samples were analyzed at
the diagnostic laboratory of the Massachusetts Institute of Technology Division of Comparative
Medicine.
Figure 5.2. The scanning electron microscope (SEM) pictures of HLGAG particles. The size, porosity, and the texture of both formulated and unformulated heparin particles were compared. A. 100% UFH particles prepared by grinding (30,000 x). B. 100% UFH prepared by spray drying (0.3% UFH solution at 12 ml/min, 120 °C inlet temperature, 100% aspirator, and 700 L/h air flow rate) (40,000 x). C. 100% LMWH dry powder prepared by spray drying (1% LMWH solution at 12 ml/min, 120 °C inlet temperature, 100% aspirator, 700 L/h air flow rate) (10,000 x). D. 100% UFH particles prepared by spray drying (1% UFH solution at 12 ml/min, 120 °C inlet temperature, 100% aspirator, and 700 L/h air flow rate) (10,000 x). The images were taken with JEOL JSM-6320 FV Scanning Electron Microscope at 1 and 5 KV.

Histology

To examine the potential pathological effects of inhaled powder on the lung histology, the lungs of rats and rabbits were harvested at the end of the experiments. The lungs were fixed
with formalin, paraffin embedded, sectioned, and stained with hematoxylin and eosin staining. The stained sections were examined with a light microscope for pathological changes.

**Activity assays**

Anti-Xa assay was used to monitor plasma heparin level. Anti-Xa assay was performed by modification of the amidolytic method of Teien and Lie with Coatest heparin test kit by using S-2222 as the chromogenic substrate (Diapharma Group, Inc. OH). The detailed procedure was described elsewhere. The concentration of LMWH in unknown samples was calculated by comparing to the calibration curve which was linear in the range of 0 – 0.7 IU/ml. The results were expressed in anti-Xa IU/ml and then in μg/ml.

Whole-blood recalcification times (WBRT) were used to indirectly determine the amount of UFH present in the blood as described. Briefly, 0.2 ml blood samples were collected into tubes containing 3.8% sodium citrate (1/9, v/v). Initially, 0.2 ml of citrated blood was added to Hemochron ACT test tubes containing glass particles (CardioMedical Products, Rockaway, NJ). Next, 0.2 ml of 0.025 M CaCl₂ was added to the test tube and the Hemochron 801 clot-timer machine (CardioMedical products, Rockaway, NJ) was immediately started. The test tube was gently mixed for 10 s, and inserted into the test well of the Hemochron 801. The time required for a clot to form was recorded. The unknown samples were compared to a standard curve, which was linear in the range of 0-4 USP units heparin/ml blood.

**Calculation of pharmacokinetic parameters**

Experimental data, expressed in μg/ml, was utilized for non-linear regression curves based on one-compartmental model by using SigmaPlot program with the method of extended least squares. From the kinetic curves, the following parameters were calculated: the area under curve (AUC expressed in μg.h.ml⁻¹), the time corresponding to the peak of maximum concentration (t_max expressed in h); the highest concentration (C_max, expressed in μg/ml); absorption rate constant (Ka expressed in h⁻¹); absorption half-life (t_{1/2} expressed in h); elimination rate constant (Ke express in h⁻¹); half-life of apparent elimination (t_{1/2e} expressed in h). The AUC (0-t) was calculated using he trapezoidal rule and extrapolated to infinity by dividing the value of the last measured concentration with the elimination rate constant.
5.3 Results of Pharmacokinetics Study

In the first rabbit model where particles were dispersed near bifurcation point, HLGAG (LMWH) particles ranging from 1 to 500 μm were first inhaled at 600 IU/kg. S.c. administration of LMWH at the same doses was included for comparison purpose. The mean plasma concentration curves derived from anti-Xa activity assay after s.c. administration and pulmonary inhalation of LMWH particles at 600 IU/kg was shown in Figure 5.3. The surprisingly fast and efficient absorption of LMWH as dry aerosol particles led us to further study the impact of particle size on the pharmacokinetics profiles of inhaled dry heparin aerosol. LMWH particles of defined sizes, ranging from 1-20, 20-53, 53-75, and 75-106 μm in geometric diameter, were tested in rabbits and the results are shown in Figure 5.3. The mean values of the main pharmacokinetic parameters derived from the plasma concentration are listed in Table 5.2. LMWH particles of different size ranges showed comparable pharmacokinetic profile. The shared pharmacokinetic features including 1) an extremely fast absorption; the absorption half-lives of all tested particle sizes are less than 8 minutes compared to more than 1 hour for s.c. administered LMWH. The \( t_{\text{max}} \) (the time to reach peak plasma activity) is around 30 minutes compared to 2-3 hours for s.c. administered LMWH. 2) A relative slow elimination process; the elimination half-lives of all tested particles at 600 IU/kg are about 2 hours which are similar to that of s.c. administered LMWH. Mean residence time (MRT) for inhaled dry LMWH particles are about half that of s.c. administered LMWH. 3) A significant bioavailability; the relative bioavailability as reflected by AUC is around 30-60% to that of s.c. administered LMWH at 600 IU/kg.

Despite the similarity in pharmacokinetics among heparin particles of different sizes, noticeable differences exist in this specific model. The major difference in pharmacokinetic profiles between particles of different size range is in the elimination phase. It was noted that particles of 53-75 μm size range is associated with a noticeably slower elimination and higher bioavailability compared to particles of other sizes (Figure 5.3; Table 5.2).
Figure 5.3. Pharmacokinetics of anti-Xa activity, expressed in µg/ml, in rabbits after pulmonary inhalation of LMWH particles and s.c. administration of LMWH. A. Pharmacokinetics of inhaled LMWH particles of various sizes at 600 IU/kg. B. Pharmacokinetics of LMWH after either s.c. administration or pulmonary inhalation at 600 IU/kg doses. C. A comparison of pharmacokinetics between pulmonary inhalation of LMWH particles and s.c. administration of LMWH at 300 IU/kg. D. A comparison of bioavailability of LMWH particle of different size range as reflected by AUC values. E. Anti-Xa Pharmacokinetics for formulated LMWH with DPPC as excipient (40% LMWH 60% DPPC) at 100, 300 and 600 IU/kg doses. F. Pharmacokinetics of LMWH after s.c. administration at 100, 300, and 600 IU/kg.
Table 5.2. The pharmacokinetics parameters of HLGAG particles derived from rabbit model 1.

<table>
<thead>
<tr>
<th></th>
<th>1-500 µm</th>
<th>1-20 µm</th>
<th>1-20 µm</th>
<th>20-53 µm</th>
<th>53-75 µm</th>
<th>53-75 µm</th>
<th>75-106 µm</th>
<th>60% DPPC 3-7 µm</th>
<th>S.C. 600 IU/kg</th>
<th>S.C. 300 IU/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IU/kg</td>
<td>IU/kg</td>
<td>IU/kg</td>
<td>IU/kg</td>
<td>IU/kg</td>
<td>IU/kg</td>
<td>IU/kg</td>
<td>IU/kg</td>
<td>IU/kg</td>
<td>IU/kg</td>
</tr>
<tr>
<td>Ka</td>
<td>5.68</td>
<td>13.62</td>
<td>19.35</td>
<td>8.16</td>
<td>11.08</td>
<td>4.65</td>
<td>5.10</td>
<td>2.86</td>
<td>0.48</td>
<td>0.58</td>
</tr>
<tr>
<td>Ke</td>
<td>0.39</td>
<td>0.22</td>
<td>0.24</td>
<td>0.36</td>
<td>0.15</td>
<td>0.46</td>
<td>0.25</td>
<td>0.35</td>
<td>0.36</td>
<td>0.23</td>
</tr>
<tr>
<td>t_{1/2a} (min)</td>
<td>7.3</td>
<td>3.1</td>
<td>2.2</td>
<td>5.1</td>
<td>3.8</td>
<td>8.9</td>
<td>8.2</td>
<td>14.4</td>
<td>87.4</td>
<td>88.5</td>
</tr>
<tr>
<td>t_{1/2e} (h)</td>
<td>1.77</td>
<td>3.20</td>
<td>2.90</td>
<td>1.95</td>
<td>4.71</td>
<td>1.51</td>
<td>2.78</td>
<td>1.98</td>
<td>1.68</td>
<td>3.28</td>
</tr>
<tr>
<td>AUC (µg.h/ml)</td>
<td>29.46</td>
<td>29.79</td>
<td>27.78</td>
<td>21.35</td>
<td>54.82</td>
<td>10.15</td>
<td>30.82</td>
<td>43.16</td>
<td>41.03</td>
<td>85.05</td>
</tr>
<tr>
<td>C_{max} (µg/ml)</td>
<td>9.16</td>
<td>6.70</td>
<td>5.48</td>
<td>6.46</td>
<td>6.58</td>
<td>4.95</td>
<td>6.62</td>
<td>10.28</td>
<td>7.09</td>
<td>10.25</td>
</tr>
<tr>
<td>t_{max} (h)</td>
<td>0.51</td>
<td>0.30</td>
<td>0.23</td>
<td>0.40</td>
<td>0.40</td>
<td>0.55</td>
<td>0.62</td>
<td>0.84</td>
<td>2.38</td>
<td>2.85</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>2.52</td>
<td>3.06</td>
<td>2.71</td>
<td>2.52</td>
<td>5.88</td>
<td>2.19</td>
<td>3.55</td>
<td>3.04</td>
<td>4.41</td>
<td>5.50</td>
</tr>
</tbody>
</table>

DPPC is a naturally occurring lung surfactant commonly used as excipient for dry aerosol delivery of molecules by pulmonary inhalation. The water-insolubility often results in sustained release of the molecules from the particles. In addition, DPPC was known to improve the absorption and aerosolization of dry aerosol particles.\(^{257,262,263}\) To investigate whether the water-insoluble DPPC can lead to a sustained release of inhaled heparin, DPPC was incorporated into LMWH particles by a single-step spray drying process. Small particles of 3-7 µm consisting 40% LMWH and 60% DPPC were obtained. This formulated LMWH preparation was subsequently tested in rabbits (Figure 5.3). As expected, a prolonged absorption and elimination was observed for the formulated LMWH particles. Meanwhile, a higher bioavailability was also observed for the formulated LMWH, a relative bioavailability of 50% was observed for the formulated LMWH compared to the 30-50% for the unformulated LMWH particles (Figure 5.3; Table 5.2). The absorption of LMWH in rabbits after inhalation appears to be dose-independent since a comparable bioavailability was observed for all three doses tested (Figure 5.3E).

The inhalation technique applied in this rabbit model only serves the purpose of proof of principle, and the results obtained with this method merely indicate that HLGAGs can be delivered by pulmonary inhalation. This method will not be able to effectively differentiate the
particles based on their aerodynamic performance due to a direct inhalation to the lower trachea of the rabbits. To compensate this defect, a rabbit model that is more reflective and informative was designed based on a ventilator-controlled respiration in rabbits. In this second model, all particles must travel the whole length of the tracheal tube and the rest of respiratory trees before reaching the deep lung (Figure 5.1B). In addition, particles will have to pass through the curvature of the trachea tube in the laryngeal region of the upper respiratory duct, which is reflective of human respiratory anatomy. Due to this added travel distance and curvature along the inhalation ducts, this method would be able to more effectively identify those particles with better aerodynamic properties for efficient lung deposition and absorption. The first experiment performed with the second rabbit model was to compare the absorption profiles of the same particles with that of the first model. The absorption profile of 53-75 μm particles was found to be rather comparable between the two methods. This result confirms that HLGAG particles can be efficiently absorbed as dry aerosol particles independent of testing systems applied.

Subsequently, this ventilator model was used to compare particles prepared with different methodology in an effort to identify the key physical and aerodynamic parameters that is required for efficient lung absorption, which could provide essential information for the eventual development of HLGAG (LMWH) particles for human inhalation. Specifically, unformulated HLGAG particles (100%) of different particle sizes (1-20, 20-53, 53-75, and 75-106 μm) as well as formulated HLGAG particles with either DPPC or lactose as excipients (40% LMWH 60% DPPC, 70% LMWH 30% lactose, 40% LMWH 60% lactose) were compared. The results of this study are presented in the Figure 5.4 and Table 5.3. The results with the second model clearly identified the differences among the powders of different composition and physical properties in terms of pharmacokinetics performance. Unformulated LMWH particles of 20-53 μm diameter showed the highest bioavailability along with a higher C_{max} and faster absorption (Figure 5.4). On the other hand, formulated particles with 70% LMWH and 30% lactose showed the least absorption. The overall pharmacokinetics profiles observed in this ventilator-controlled model are similar to that of first rabbit model where the powder is delivered directly to the lower tracheal tube.
Figure 5.4. The pharmacokinetics of anti-Xa activity for particles of different size and composition after ventilator-assisted inhalation in rabbits.

| Table 5.3. Pharmacokinetics parameters for HLGAG particles derived from the rabbit ventilator model.  

<table>
<thead>
<tr>
<th>Parameter</th>
<th>1-20 μm</th>
<th>20-53 μm</th>
<th>53-75 μm</th>
<th>75-106 μm</th>
<th>60% DPPC 40% LMWH</th>
<th>70% lactose 30% LMWH</th>
<th>60% lactose 40% LMWH</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_a$</td>
<td>4.73 ± 1.83</td>
<td>10.41 ± 1.91</td>
<td>3.72 ± 0.32</td>
<td>5.15 ± 0.14</td>
<td>6.23 ± 3.53</td>
<td>3.95 ± 0.54</td>
<td>7.55 ± 1.55</td>
</tr>
<tr>
<td>$K_p$</td>
<td>0.50 ± 0.11</td>
<td>0.23 ± 0.05</td>
<td>0.36 ± 0.07</td>
<td>0.63 ± 0.08</td>
<td>0.23 ± 0.06</td>
<td>0.72 ± 0.06</td>
<td>0.82 ± 0.05</td>
</tr>
<tr>
<td>$t_{1/2a}$ (h)</td>
<td>0.19 ± 0.05</td>
<td>0.07 ± 0.01</td>
<td>0.19 ± 0.02</td>
<td>0.13 ± 0.00</td>
<td>0.16 ± 0.09</td>
<td>0.18 ± 0.02</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>$t_{1/2p}$ (h)</td>
<td>1.52 ± 0.23</td>
<td>3.15 ± 0.65</td>
<td>2.03 ± 0.40</td>
<td>1.12 ± 0.14</td>
<td>3.20 ± 0.84</td>
<td>0.97 ± 0.08</td>
<td>0.85 ± 0.05</td>
</tr>
<tr>
<td>AUC (IU.h/ml)</td>
<td>1.82 ± 0.34</td>
<td>4.97 ± 1.45</td>
<td>2.71 ± 0.49</td>
<td>1.08 ± 0.14</td>
<td>3.36 ± 0.14</td>
<td>0.51 ± 0.02</td>
<td>0.72 ± 0.20</td>
</tr>
<tr>
<td>$C_{max}$ (IU/ml)</td>
<td>0.56 ± 0.14</td>
<td>1.07 ± 0.04</td>
<td>0.66 ± 0.10</td>
<td>0.45 ± 0.03</td>
<td>0.68 ± 0.21</td>
<td>0.25 ± 0.00</td>
<td>0.41 ± 0.07</td>
</tr>
<tr>
<td>$t_{max}$ (h)</td>
<td>0.61 ± 0.13</td>
<td>0.38 ± 0.03</td>
<td>0.70 ± 0.00</td>
<td>0.47 ± 0.01</td>
<td>0.73 ± 0.36</td>
<td>0.53 ± 0.03</td>
<td>0.34 ± 0.05</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>2.13 ± 0.31</td>
<td>3.33 ± 0.31</td>
<td>3.50 ± 0.23</td>
<td>1.89 ± 0.36</td>
<td>3.47 ± 0.47</td>
<td>1.49 ± 0.09</td>
<td>1.38 ± 0.15</td>
</tr>
</tbody>
</table>

$^1$ mean ± SE.

In this particular model, differential absorption profiles were observed for particles of different sizes and composition (Figure 5.4; Table 5.3). Unformulated HLGAGs prepared by grinding and sieving showed generally better pharmacokinetics performances compared to the spray dried counterparts. This is consistent with the observation that substantial amount of spray dried particles adhered to the trachea tube at the time of inhalation. In the unformulated HLGAG particles prepared by grinding and sieving, particles with size ranging from 20 to 53 μm showed
the most efficient absorption (Figure 5.4). The formulated particles also showed different pharmacokinetic profiles depending on the composition and preparation procedure. Particles with 70% LMWH and 30% lactose showed least absorption compared to the particles with 40% LMWH and 60% lactose despite similar spray-drying procedures (Figure 5.4 and Table 5.3). HLGAG particles with DPPC as excipient appear to be more efficiently absorbed than those particles with lactose as excipient (Table 5.3). Thus, the ventilator model appear to be able to differentiate the particles based on their dispersibility and aerodynamics, which is indicative of how a particle will perform in human application.

To investigate whether this efficient absorption of LMWH is species dependent, HLGAG particles were further tested in a rat model. In the rat model, pulmonary inhalation of LMWH particles resulted in similar pharmacokinetics compared to that observed in rabbit models (Table 5.4; Figure 5.5A,B). However, in contrast to that of rabbit data, the bioavailability of LMWH in rat model seems to be dose-dependent. A higher relative bioavailability is achieved at higher dose (88% bioavailability at 600 IU/kg) compared to that of lower doses (less than 30% for 100 and 300 IU/kg doses). Both formulated and unformulated UFH dry powder gave significant absorption after inhalation as reflected by the prolonged whole blood clotting assay (Figure 5.5C,D). These results suggest that pulmonary delivery of HLGAG particles can be achieved in different species despite some different characteristics of absorption.

To evaluate if the absorption of dry HLGAG aerosols by pulmonary inhalation induce pulmonary absorption resistance. The rabbits were inhaled with unformulated LMWH particles (53-75 μm in size) consecutively for 3 days at 600 IU/kg. Comparable pharmacokinetics parameters were observed during the 3-day treatment (Figure 5.6). No significant change in pharmacokinetic parameters was observed, suggesting that heparin particles can be delivered repetitively without eliciting significant absorption resistance.
Figure 5.5. The pharmacokinetics of pulmonary delivered LMWH and UFH in rats. A. Anti-Xa activity pharmacokinetics of pulmonary inhaled formulated LMWH (40% LMWH 60% DPPC) at 100, 300, and 600 IU/kg in rats. B. Anti-Xa activity pharmacokinetics of s.c. administered LMWH at 100, 300, and 600 IU/kg in rats. C. Pharmacokinetics of pulmonary delivered UFH as unformulated dry powder (1-500 μm) (12 mg/kg) or formulated nonporous small particles (1-3 μm) with DPPC as excipient (60% UFH 40% DPPC) (10 mg/kg) in rats. D. A comparison between s.c. injection of UFH at 8 mg/kg and pulmonary inhalation of unformulated UFH (1-500 μm) at 12 mg/kg.
Table 5.4. Pharmacokinetic parameters after inhalation and s.c. administration in rats.

<table>
<thead>
<tr>
<th></th>
<th>Powder 600 IU/kg</th>
<th>Powder 300 IU/kg</th>
<th>Powder 100 IU/kg</th>
<th>S.C. 300 IU/kg</th>
<th>S.C. 600 IU/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_a$</td>
<td>0.0057</td>
<td>0.016</td>
<td>0.0098</td>
<td>0.0048</td>
<td>0.0054</td>
</tr>
<tr>
<td>$K_e$</td>
<td>0.0034</td>
<td>0.009</td>
<td>0.0079</td>
<td>0.004</td>
<td>0.0041</td>
</tr>
<tr>
<td>$t_{1/2a}$(h)</td>
<td>2.03</td>
<td>0.72</td>
<td>1.18</td>
<td>2.4</td>
<td>2.14</td>
</tr>
<tr>
<td>$t_{1/2c}$(h)</td>
<td>3.40</td>
<td>1.28</td>
<td>1.46</td>
<td>2.89</td>
<td>2.81</td>
</tr>
<tr>
<td>AUC(μg.h/ml)</td>
<td>110.4</td>
<td>15.0</td>
<td>5.0</td>
<td>69.2</td>
<td>125.0</td>
</tr>
<tr>
<td>$C_{max}$ (IU/ml)</td>
<td>10.7</td>
<td>3.9</td>
<td>1.1</td>
<td>15.3</td>
<td>6.9</td>
</tr>
<tr>
<td>$t_{max}$ (h)</td>
<td>3.74</td>
<td>1.37</td>
<td>1.9</td>
<td>3.8</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Figure 5.6. The pharmacokinetics of unformulated dry LMWH particles (53-75 μm) during 3 consecutive treatment by pulmonary inhalation.

To investigate the mechanism of absorption after pulmonary inhalation of HLGAG particles, the lungs of rabbits were lavaged at the indicated times after inhalation, and the concentration of HLGAG in the lavage fluid was determined with anti-Xa activity assay. Instillation of liquid HLGAG at the same dose was included for reference. To our surprise, the LMWH in the lavage fluid disappeared precipitously after inhalation and the level of LMWH in the lavage fluid reached that of base line within about 1 h (Figure 5.7A). The calculated $t_{1/2}$ for elimination from the lavage fluid is less than 10 minutes. When converted to total amount of heparin in the fluid, approximately 90% of the LMWH disappeared from the lung air-duct surface in the first half hour. This fast disappearance of LMWH coincides with the rapid
appearance of LMWH in plasma (Figure 5.7A; Figure 5.3). Furthermore, the homogenized lung tissue and macrophage cell pellets from centrifugation contained little LMWH as observed with anti-Xa assay. In sharp contrast, the LMWH level in the lavage fluid decreased at a much slower rate after instillation of the liquid LMWH at the same dose (Figure 5.7B). Substantial amount of LMWH remained in the lavage fluid even 8 h after instillation in rabbits. The slow decrease of anti-Xa activity in the lavage fluid was accompanied with slow rise in the plasma anti-Xa activity after instillation (Figure 5.7B).

![Graph A](image1.png) ![Graph B](image2.png)

**Figure 5.7.** A. The time courses of LMWH levels in lavage fluid and plasma after pulmonary inhalation of LMWH particles (unformulated 53-75 μm) at a dose of 600 IU/kg. Total amount of LMWH (mg) in lavage fluid or plasma was used for comparison. B. The time course of LMWH levels in lavage fluid and plasma after instillation of LMWH at 600 IU/kg dose.

Hematological analysis of blood samples withdrawn before and after the experiments revealed no significant difference in the parameters examined, suggesting there is no significant adverse effects on hematology by pulmonary inhalation treatment or multiple blood withdraws. In addition, histology examination of the lungs of rabbits after the pulmonary inhalation showed no signs of pathological changes. No signs of morbidity such as loss of weight or labor of breathing were observed in animals, during and after the treatment. Combined together, these results indicate that pulmonary inhalation of HLGAG particles represents a safe alternative administration route for heparin.
5.4 Discussion

The present study demonstrated for the first time that HLGAGs can be efficiently delivered as dry aerosol particles via pulmonary inhalation. The pharmacokinetic profiles of inhaled LMWH are similar to that of s.c. administration and distinct from that of instillation or inhalation of liquid heparin solution. This efficient absorption of HLGAGs after pulmonary inhalation appeared to be species and model systems independent. Both rats and rabbits showed similar pharmacokinetics profiles after inhalation. The relative bioavailability of inhaled heparin is determined to be 30-60% compared to s.c. administration. Compared to s.c. administration, pulmonary inhalation of dry heparin particles is associated with faster absorption. The fast absorption of inhaled dry heparin aerosol can be very beneficial during emergency conditions such as acute coronary syndrome. Another potential advantage of dry heparin particles as demonstrated in this study is that the pharmacokinetic parameters of inhaled heparin can be adjusted by manipulating either the physical properties or composition of the particles, which conceivably will allow generating of formulations customized for specific clinical applications. Furthermore, a lack of resistance after repetitive pulmonary inhalation of dry heparin particles allows for a long-term use of pulmonary delivery of heparin for prophylaxis of thrombosis.

Deep lung is widely believed to be responsible for the majority of absorption after pulmonary delivery of drugs. It is well established that a geometric diameter of 1-5 μm is required for efficient deep lung deposition of inhaled particles.\textsuperscript{264} Alternatively, inhaled particle must possess a very narrow range of "aerodynamic diameters (1-3 μm) to efficiently deposit into the deep lung. Aerodynamic diameter is the geometric diameter a particle appears to possess on the basis of its in-flight speed, were it assumed to be spherical and to possess a mass density of 1 g/cm\(^3\); stated differently, the geometric diameter of a spherical particle possessing unit mass density (1 g/cm\(^3\)) is equivalent to its aerodynamic diameter.\textsuperscript{264,265} Thus, aerodynamic diameter has been used for several decades to quantify an aerosol particle's inherent propensity to deposit in the lungs, essentially independent of its shape, mass density, and (in principal) geometric size.\textsuperscript{264,266} Numerous experimental and theoretical studies have demonstrated that particles of mean aerodynamic diameter of 1-3 μm deposit maximally in the deep lung (alveoli). Particles with aerodynamic diameter between 8-10 μm are maximally deposited in the tracheobronchial
surface. Particles possessing aerodynamic diameter smaller than 1 μm are mostly exhaled \(^{264}\). The present study showed efficient absorption of heparin particles with various aerodynamic diameters (1-35 μm), suggesting that the absorption of heparin may not be limited to the deep lung. However, it is shown in this study that particles of certain sizes and composition fare better than others in the ventilator controlled rabbit inhalation model (Figure 5.4). It is concluded from this study that the efficiency of lung delivery for dry aerosolized HLGAGs is determined by multiple factors including particle sizes, surface texture, density, composition and etc. Unformulated LMWH and 40% LMWH 60% lactose particles showed higher bioavailability compared to 70% LMWH 30% lactose particles (Figure 5.4). Respiratory tract may also be involved in the absorption of heparin. However, it is necessary to point out that the experimental model applied here may not fully predict the outcome in human application. One important observation from this study is that the fast, efficient absorption of the inhaled dry HLGAG particles and their predictable pharmacokinetics are distinctly different from the pulmonary delivery of liquid HLGAGs by either inhalation or instillation \(^{242,251,252}\).

The present study showed that a therapeutic level of HLGAGs can be achieved by pulmonary delivery of dry aerosolized HLGAGs at a dose comparable to that of s.c. administration. Additionally, the dry HLGAG aerosol offers the advantages of convenient, safe, and noninvasive self-administration on an outpatient basis. Consequently, pulmonary inhalation of dry aerosolized heparin represents a breakthrough in pulmonary delivery of HLGAG-based therapeutics such as heparin and offers a novel alternative route for heparin administration to the existing injection method.
Chapter 6

Investigations into Clinical Applications of Inhaled HLGAGs

Summary

In the previous chapter, it was shown that HLGAGs can be efficiently delivered to the lung to achieve both high local concentration and efficient systemic absorption. In this chapter, the potential applications for such novel delivery approach were studied. HLGAGs, especially heparin and LMWHs, have long been known to be effective in preventing thrombosis, human leukocyte elastase induced emphysema, and tumor lung metastasis. To investigate whether the high local concentration in the lung and efficient systemic absorption of pulmonary delivered dry HLGAG aerosols can be used to treat diseases in circulation system and in the lungs, pulmonary inhaled HLGAGs were tested in several animal disease models. Firstly, the high local concentration achieved after inhalation of HLGAG powder was demonstrated to be effective in preventing HLE induced acute and chronic emphysema in rat emphysema models. Secondly, pulmonary inhalation of dry HLGAG aerosol was shown here to be effective against thrombosis in two animal thrombosis models as a result of efficient absorption of HLGAGs into blood circulation system. Lastly, a significant inhibition of lung metastasis of melanoma cells was observed when HLGAGs were directly inhaled to the lung of mice. These results provided the first experimental evidence for therapeutic application of pulmonary delivered HLGAGs as dry aerosol. These findings have resulted in one patent application and several papers in preparation.
6.1 Prevention of Emphysema with Inhaled Dry Aerosol HLGAGs

6.1.1 Introduction

Emphysema is a condition of the lung characterized by abnormal permanent enlargement of the airspaces distal to the terminal bronchiole, accompanied by destruction of their walls, and without obvious fibrosis. Depending on the anatomic nature of the lesion, emphysema includes four types: (1) centriacinar, (2) panacinar, (3) paraseptal, and (4) irregular. Centriacinar and panacinar emphysema are the most clinically relevant types (Figure 6.1). Emphysema is a common disease. In one study, there was a 50% combined incidence of panacinar and centriacinar emphysema at autopsy, and the pulmonary disease was considered to be responsible for death in 6.5% of these patients. There is a clear-cut association between heavy cigarette smoking and emphysema, and the most severe type occurs in men who smoke heaviy. Although details of the genesis of the emphysema remain unsettled, the most plausible hypothesis to account for the destruction of alveolar walls is the protease-antiprotease mechanism. This hypothesis is supported by several lines of clinical and experimental observations. The protease-antiprotease theory argues that alveolar wall destruction results from an imbalance between human leukocyte elastase (HLE) and antiprotease. In normal lungs, HLE are counter balanced by elastase inhibitors such as α1-antitrypsin (α1-AT). The balance was tipped toward increased elastase activity as a result of chronic inflammation and smoking. The result of this unbalanced HLE activity is the destruction of elastic fiber and other structural proteins, which lead to emphysema. Due to loss of elasticity, emphysematous lungs cannot retract efficiently and this lead to the enlarged alveoli and respiratory ducts. Patients of emphysema suffer from dyspnea and difficulty in exhaling.

The principle elastase involved in emphysema formation is derived from infiltrated leukocytes (neutrophils), and instillation of human leukocyte elastase into the lung produce emphysema in animal experiments. HLE, a highly basic protein, is one of the most destructive enzymes in the body, having the ability to degrade many structural proteins such as type I, II, III and IV collagens, and elastin. The protease-antiprotease hypothesis also explains the early onset of emphysema in patients with protease inhibitor α1-AT deficiency.
Figure 6.1 The pathological changes representative of two major emphysema types: centrilobular (A) and panacinar (B) emphysema modified from Contran et al.²⁵⁵

For this reason, much effort has been devoted to the development of novel elastase inhibitors in hope of preventing and curing this devastating disease. A wide variety of inhibitors have been developed for HLE. These include active site-directed irreversible inhibitors, enzyme-activated irreversible inhibitors (suicide inhibitors), and tight binding reversible inhibitors.²⁶⁷ It is conceivable that an ideal drug would be naturally occurring HLE inhibitors that can be targeted to the lower respiratory tract. Studies have shown that HLGAGs are potent HLE inhibitors that are expected to be safe for lung application. $K_i$ values ranged from 40 nm to 100 μM and were found to be inversely correlated to the chain length of the oligosaccharides. Desulfated HLGAGs are found to be less active.²⁶⁹ It was shown that the interaction between HLGAGs and HLE occurs by the formation of electrostatic bonds between the negatively charged sulfate groups and the positively charged groups in the cationic enzyme.²⁷¹ In vitro study showed an $IC_{50}$ of ngs for heparin in inhibiting HLE and this elastase inhibitory activity of heparin is largely independent of its anticoagulant activity.²⁹ In vivo study in animal models further confirmed the protective effect of injected heparin against HLE induced experimental emphysema.²⁶⁷,²⁷² The research has been limited to animal research partly due to concerns over the systemic side effects of heparin after administration of heparin by injection.

The previous chapter demonstrated that HLGAGs can be efficiently delivered to the lung without eliciting obvious side effects. In addition, inhaled heparin can reach the deep lung and achieve high local concentration, and the deep lung is the site of the tissue injury caused by elastase. For these reasons, it is expected that pulmonary inhaled dry aerosol HLGAG particles
can efficiently prevent the emphysema caused by HLE. To test this hypothesis, both acute and chronic rat emphysema was established.

6.1.2 Methods

In acute emphysema model, LMWH particles at 600 IU/kg or UFH particles at 12 mg/kg were inhaled to the rats 5 minutes prior to instillation of 250 μg of human sputum leukocyte elastase via the trachea. The rats were kept head up at a 30 degree slope for 30 minutes. The incision was sutured and the rats were allowed to recover. 24 hours later the rats were euthanized; the lungs were harvested en bloc and lavaged. The level of hemoglobin in the lavage fluid was determined by a colorimetric assay kit from Sigma.

Since human emphysema is a chronic disease, a chronic rat model was also established to test the efficacy of inhaled LMWH in preventing the HLE induced emphysema. The rats were treated with HLE as described. In contrast to the acute emphysema model, HLE and LMWH treated rats were allowed to recover and live for 8 weeks. At the end of 8 weeks, the animals were euthanized by exsanguinations and the lungs were fixed in 10% formalin at 20 cm H₂O pressure for 24 hours. Lung volume was measured before and after fixation by method of water displacement. At the end of fixation, histological sections were prepared by systemic sampling method, and the histological sections were stained with hematoxylin and eosin (H&E) staining. The extent of emphysema for lung tissues was evaluated under light microscope by measuring the mean linear intercept Lₙ. A larger Lₙ value reflects longer distance between the two adjacent alveolar walls that indicates the destruction of alveolar walls. Also, the surface area of the air-alveoli interface was calculated according to Dunnill.
Figure 6.2. A. The protection of acute injury induced by human sputum leukocyte elastase (HLE) in lung tissue by pulmonary inhalation of heparin particles. Formulated UFH (60% UFH 40% DPPC, 1-3 μm) at 12 mg/kg or formulated LMWH particles (40% LMWH 60% DPPC, 3-7 μm) at 600 IU/kg were inhaled to rats 5 minutes prior to instillation of 0.25 ml of HLE (250 μg). Rats were sacrificed 24 hours later, the lungs were harvested and lavaged. The hemoglobin level in the lavage fluid was assayed. Control group received no heparin was included for comparison. B. Represenative pictures of lungs from HLE treated rats and rats received both HLE and LMWH inhalation. It is noted that the distribution pattern of hemorrhage was changed as a result of HLGAG inhalation. C. Mean linear intercept calculated for the lungs from rats received no treatment, HLE alone and HLE plus LMWH (3-7 μm) inhalation in chronic emphysema study. D. Alveolar surface area calculated for lungs of rats received no treatment, HLE alone, and HLE plus inhalation of LMWH in chronic emphysema study. E, F, I are representative pictures of lungs from normal control, HLE, HLE and LMWH inhalation treated rats (200 x).
6.1.3 Results and Discussion

Figure 6.2 shows the results from acute and chronic emphysema animal studies. Pulmonary inhalation of the dry aerosolized particles containing 40% LMWH effectively protected the lungs from HLE elicited injury as reflected by the reduced hemoglobin level in the lavage fluid (Figure 6.2A). In addition, the total number of the cells and total protein concentration in lavage fluid was also reduced by inhalation of heparin particles. Most significantly, the pattern of hemorrhage was distinct from that of control group. The hemorrhagic spots scattered randomly throughout the lung surface in the control group while hemorrhage was limited only to the upper lobes of the dry aerosol treated group with no obvious hemorrhage found at the base and lower lobes of the lungs (Figure 6.2B). This is consistent with the notion that particles deposit preferably to the base of the lung due to more efficient ventilation. The neutrophil counts in the treated and control groups are comparable. Histological examination confirmed gross observations. Histological sections showed hemorrhage and exudate throughout the lungs of rats in the HLE treated group, but these pathological changes are limited to the upper lobes of lungs in the inhaled groups.

Similarly, inhalation of HLGAGs before instillation of HLE significantly prevented the chronic injury in rat lungs induced by HLE (Figure 6.2C, D, E, F). The $I_n$ and alveolar surface area values derived from inhalation and HLE treated group are comparable to that of control group, both are significantly different from the HLE treated group (Figure 6.2C, D). Microscopic examination of the histology sections from the lungs of differently treated rats revealed similar changes, namely, more destruction in the alveoli of the HLE treated lungs compared to rats received no HLE treatment or rats received both HLGAG inhalation and HLE treatment (Figure 6.2E, F, I).

These results provided experimental evidence for the effectiveness of local therapy and prevention of emphysema with inhaled HLGAGs as dry aerosol particles. It is predicted that improved efficacy can be achieved by either improving the aerodynamics of dry aerosol particles or increasing the potency of HLE-inhibitory HLGAG species.
6.2 Inhibition of Thrombosis by Inhaled HLGAGs

6.2.1 Introduction

Under normal physiologic circumstances, a haemostatic balance is maintained by various interacting elements that include endothelial cells, platelets, adhesive proteins and coagulation/fibrinolysis system. Thrombotic processes may occur in any part of the cardiovascular system, including veins and arteries. An insult to the endothelium trigger the adhesion of platelets and activation of blood coagulation cascade, including activation of factors X and II, leading to the formation of clot and thrombosis (Figure 1.4). As stated in the PART I, HLGAGs, such as heparin and LMWH containing AT-III binding pentasaccharide sequence, are widely used in clinic as anti-coagulation and antithrombotic agents. LMWHs have been increasingly used for long-term prevention of thrombosis by means of daily s.c. injection. Prolonged injection is associated with local irritation and ulceration that leads to poor compliance from patients. Thus, it is of great practical value to develop non-invasive, convenient delivery method for heparin application. The pulmonary delivery of heparin as dry aerosol powder was shown in the present study to be pharmacokinetically efficient. To further question whether this novel delivery strategy bears practical value, animal thrombosis models were used to address the question.

6.2.2 Method

Rabbit thrombosis model

The antithrombotic effects of inhaled LMWH was evaluated by Wessler’s stasis model adapted for the rabbit using RVV as the thrombogenic stimulus. The procedure was performed as described. Briefly, 30 min. and 2 h after pulmonary inhalation of unformulated LMWH particles (53-75 μm) at 600 IU/kg to the anesthetized rabbits (n=2), RVV (0.05 U/kg) were injected via marginal ear vein in 10 seconds. 2 minutes after RVV injection, the pre-exposed jugular veins at both sides were ligated. 10 minutes after ligation, the thrombus (if formed) was removed and weighed wet. Control rabbits received no heparin was included.

Rat thrombosis model

The antithrombotic effects of inhaled heparin were evaluated by Wessler’s stasis model adapted for the rat using RVV as the thrombogenic stimulus. The procedure was
performed as described\textsuperscript{277}. Briefly, two hours after pulmonary inhalation of 40% LMWH 60% DPPC powder (3-7 μm) after anesthesia, laparotomy was performed. A vena cava segment was isolated between the left renal vein and the iliac veins. All the side branches of vena cava were ligatured and the RVV (0.03 U/kg) was administered via the penile vein. Thirty seconds later, the vena cava was ligatured by cotton threads just below the left renal vein and above the iliac veins. Ten minutes later, the thrombus (if formed) was removed and weighed wet.

6.2.3 Results and Discussion

In the rabbit jugular vein stasis model\textsuperscript{193}, thrombosis was completely prevented when Russel’s viper venom (0.05 U/kg, RVV) was injected either 30 min or 2 h after inhalation of LMWH particles at 600 IU/kg dose (Figure 6.3). The antithrombotic action of inhaled LMWH is consistent with the observed anti-factor Xa pharmacokinetics profiles after pulmonary inhalation of unformulated LMWH particles.

![Figure 6.3](image-url)

**Figure 6.3.** A. The antithrombotic effect of inhaled HLGAG particles (100% unformulated LMWH particles of 53-75 μm size) at 600 IU/kg in a rabbit stasis thrombosis model. No significant thrombosis formation was found when RVV was injected either 30 min or 2 h after inhalation of HLGAG. B. The antithrombotic effect of inhaled HLGAGs (40% LMWH 60% DPPC, 3-7 μm in size) in rat thrombosis model. Error bars indicate SEM.

In the rat thrombosis model, despite relatively lower bioavailability of HLGAG by inhalation at 300 IU/kg compared to that of s.c. administration, it effectively inhibited the formation of thrombus (Figure 6.3B). At 600 IU/kg, pulmonary inhalation of HLGAGs...
completely prevented the formation of thrombus. The antithrombotic effect of inhaled HLGAGs is closely related to the plasma heparin level as reflected by anti-factor Xa activity. This effect of inhaled heparin is very comparable to that of s.c. injection of LMWH. These results indicate that pulmonary inhalation of heparin in animals is not only pharmacokinetically efficient but also pharmacodynamically effective.
6.3 Inhibition of Lung Metastasis by Pulmonary Inhalation of HLGAGs

6.3.1 Introduction

Tumor metastasis to the target organ such as lungs involves multiple distinct steps that are not completely understood (Figure 6.4). After being released from the primary tumor into the blood circulation, tumor cells come to rest in a capillary of a distant site where metastatic tumor ultimately forms. It was hypothesized that after lodging in the capillary site of target organ, tumor cells trigger local coagulation components and the formation of a fibrin clot is believed to protect the tumor cells from destruction by the host defense \(^{132,143}\). Additional mechanism involved including the cell surface molecules that mediate adhesion processes. As mentioned in the chapter I, HLGAGs such as heparan sulfate has been shown to be involved in adhesion molecules such as selectins mediated process as well \(^{93,278}\). The interruption of P-selectin – tumor cell surface carbohydrate interaction by heparin was recently shown to inhibit human tumor cell metastasis \(^ {95}\). Therefore, HLGAGs may potentially inhibit tumor metastasis by interfering both processes during the initial stages of metastasis. Consistent with this notion, several studies have shown that HLGAGs, including heparin, heparan sulfate and other sulfated polysaccharides, can inhibit tumor metastasis \(^ {91,135,136,140,141,189,279}\). Similarly, chapter 5 of this thesis research clearly demonstrated that HLGAG fragments with defined sequence information can potently inhibit tumor metastasis. Aforementioned inhibition of metastasis by heparin was achieved by injection method. It is interesting to find out whether inhibition of metastasis can also be achieved by pulmonary inhalation of anti-tumor HLGAGs. Since heparin is well known for its inhibition of B16 melanoma metastasis, dry aerosolized heparin was chosen as the model drug in inhibition of B16 melanoma lung metastasis \(^ {189}\).

6.3.2 Methods

Heparin was inhaled to mice prior to the injection of B16BL6 melanoma cells via tail vein. The mice were placed inside a container that is attached to a dry powder sprayer, which was loaded with HLGAG powder with particle sizes ranging 1-200 \(\mu m\) (Figure 6.5A). In treatment groups, 8 mice were inhaled with either UFH or LMWH for 10 minutes prior to tail vein injection of tumor cells. Control groups receiving B16 melanoma cells along or tumor cell suspension containing LMWH to directly test whether the HLGAGs used here inhibit metastasis.
of B16 cells were included. Mice were sacrificed and lungs harvested 12 days after injection. Lung metastasis was evaluated by counting the number of nodules on the lung surface with the assistance of a dissection microscope. The blood was withdrawn 10 min after inhalation and tested for the presence of heparin by means of anti-Xa activity.

Figure 6.4. A pictorial description of steps that are involved in the formation of a distant metastasis.

6.3.3 Results and Discussions

Consistent with the reported literature, mice receiving i.v. B16 melanoma cells doped with LMWH had significantly less lung metastasis compared to the control group receiving tumor cells alone (Figure 6.5B). The high concentration of LMWH in the cell suspension (2 mg/ml) virtually eliminated lung metastasis by B16 melanoma cells, confirming that the heparin applied in the study is intrinsically anti-metastasis. Mice inhaled UFH prior to tumor cell
injection also showed less metastasis and the inhibition is proportional to the period of time for inhalation. A similar inhibition of lung metastasis by B16 cells was observed for mice inhaled LMWH. The level of the heparin in the blood 10 min after inhalation was determined to be at about 25 μg/ml (compared to ≈ 500 μg/ml in the i.v. injected LMWH group). Therefore the extent of inhibition seems to be proportional to the level of heparin achieved in the plasma.

In summary, this research demonstrated that a significant inhibition of tumor lung metastasis can be achieved by pulmonary delivery of dry aerosolized HLGAGs.

**Figure 6.5** Inhibition of lung metastasis by pulmonary inhalation of heparin. A. 2x 10^5 B16 melanoma cells in 0.2 ml PBS were injected via the tail vein. Heparin inhalation groups were put in a customized device and inhaled dry heparin aerosol for 10 minutes as described. The particle sizes range 1-200 μm. B. Results of lung metastasis in control groups received either no heparin treatment or 20 mg/kg LMWH in cell suspension, and inhalation treatment groups.
PART V  IMPLICATIONS
Chapter 7.  Conclusions and Significance

7.1 Conclusions of the Research

Heparin-like glycosaminoglycans are a family of information dense and structure complex biopolymers with many untapped application potentials. Due to lack of the tools, past research only indirectly suggests HLGAGs as being important regulators of tumor progression, and the mechanism of which was poorly understood. An understanding of the roles for HLGAGs in cancer biology provides a novel strategy for development of cancer therapy. As macromolecules, the conventional injection method of administration of HLGAG-based therapeutics is associated with side effects and poor patient compliance. This thesis research began with the establishment of important roles for HLGAGs in tumor progression and delineation of the mechanism for tumor regulatory effects of HLGAG fragments, and completed with development of a novel noninvasive and efficient delivery strategy for HLGAG-based therapeutics. The research goals accomplished are summarized below.

- Calcium bindings sites and coordinating amino acids in hep I were fully identified and characterized. By using biochemical tools, two calcium binding sites were identified and located. Subsequently, by means of site-directed mutagenesis and biochemical analysis, individual amino acids involved in calcium binding were identified. The relative importance of each binding sites and amino acid residues were compared, and mechanism of action for calcium in each binding sites were provided.

- The importance of HLGAGs in tumor biology was first established by in vitro cell culture studies with heparinases I and III as tools. The inhibitory effect of heparinase III on tumor cell proliferation, invasion and adhesion was identified. In vivo animal tumor experiments clearly identified distinct, opposing effects of heparinase I and III on tumor progression. HLGAG fragments derived from heparinase III treatment of tumor cells were found to be potent inhibitors of tumor progression, which are structurally distinct from the fragments derived from heparinase I treatment of tumor cells. The mechanism of action was provided as multiple levels. At cellular level, immunohistochemical study showed that both tumor and endothelial compartments were affected by the HLGAG fragment treatment. At molecular level, FGF signaling
was identified as the principal pathway that is modulated by HLGAG treatment. At animal level, the inhibition of FGF signaling was confirmed in a rat corneal angiogenesis assay.

- In addition to the current clinical applications, more therapeutic applications for HLGAGS are likely to be discovered in the years to come. This is exemplified by the result from this thesis research; HLGAGs of distinct sequence information can be used to treat cancer. To maximize the therapeutic index and minimize the side effects associated with injection method, a novel noninvasive and convenient delivery strategy was developed in this thesis research. By using anticoagulant HLGAGs as model drugs, it was shown that an efficient absorption can be achieved by pulmonary inhalation of HLGAGs as dry aerosol powder. The research presented here also provided mechanistic aspect of absorption for inhaled HLGAGs. This novel strategy was further proven to be practical values in animal experiments where local and systemic therapeutic effects of inhaled HLGAGs are demonstrated.

- The relationship between particle properties and inhalation efficiency was investigated. In the model system tested, it appears that HLGAG aerosol can be absorbed outside alveoli in the air-conducting bronchial tree since particles with aerodynamic diameter greater than 3 μm showed efficient absorption. This is an important observation since most inhaled compounds are primarily absorbed in the deep lung.

7.2 Significance: A Framework for Further Studies on Therapeutic Discovery and Delivery of HLGAGs

The research presented in this thesis research is centered around the elucidation of the biological functions of HLGAGs in cancer and the development of a novel delivery strategy for optimal application of such therapeutic HLGAGs. Studies have been performed to establish a fundamental role for HLGAGs in regulating tumor progression. Hep III and its derived HLGAG fragments are shown to potently inhibit both primary tumor growth and secondary metastasis, and this is in contrast to that of hep I and hep I generated fragments. Further, the composition and structures of two HLGAG fragments generated by hep I and III were found to be distinctly different. An important conclusion drawn from the study is that heparan sulfate chains at the
tumor cell surface are cryptic modulators of tumor progression, activated to be either activators or inhibitors upon distinct enzymatic degradation of cell surface HS chains. Just as collagenases clip the proteinaceous compartment of the ECM, serving either to increase tumor growth (eg., breakdown of the basement membrane)\textsuperscript{237,238} or to inhibit tumors (eg., the formation of endostatin from collagen XVIII)\textsuperscript{185,186}, the polysaccharide compartment exhibits a similar phenomenon. Importantly, like the proteolytically cleaved collagen fragment endostatin, distinct HLGAG oligosaccharides upon release by enzymatic cleavage from the tumor cell surface can serve as potent inhibitors of tumor progression. Thus, the present study not only allows a new paradigm of how the polysaccharides modulate tumor growth and metastasis, but it identifies a novel therapeutic target by providing a framework towards the development of HLGAG-based novel anti-cancer molecules.

The implication for pulmonary delivery of HLGAGs is varied and profound. Heparin and LMWHs are currently widely used as anticoagulant and anti-thrombotic agents by means of injection, and this has severely limited wider application of these highly effective medicines on an outpatient basis. The research finding of this thesis research provided foundation for eventual clinically applicable inhalation formulation, which will greatly expand the application of heparins as anti-coagulant and anti-thrombotic medicines and improve patient compliance virtually due to the convenience of inhalation. Furthermore, HLGAGs, especially heparins, are being investigated for non-anticoagulant applications. Such potential applications are found in cancer treatment, asthma therapy, emphysema prevention, acute coronary syndrome treatment, etc. Due to a direct deposition to the lung tissue, pulmonary inhalation of HLGAGs can be especially valuable in treating respiratory diseases due to the high therapeutic ratio and local concentration.

7.3 Recommendations for Future Research

Two major themes emerged from this thesis research are the discovery of tumor inhibitory HLGAG fragments on the tumor cell surface and development of an efficient pulmonary delivery strategy for HLGAG-based therapeutics.

In the first theme, the present research provided foundation for:
1) Isolation and sequencing of tumor inhibitory HLGAG fragments. By scaling up production of hep III derived HLGAG fragments, sufficient amount of HLGAG fragments should be available for isolation, separation, and testing of individual HLGAG chains for the anti-tumor activities. Once the inhibitory fragment(s) is identified, sequencing can be accomplished with the sequencing methodology developed in this laboratory. Once the sequence of specific inhibitory sequence is known, a clear structure-function relationship for HLGAGs in tumor biology can be elucidated.

2) Pharmaceutical development of HLGAG-based cancer therapeutics. Large-scale production of inhibitory HLGAG sequence(s) can produce novel anti-cancer HLGAG-based therapeutics. The production of HLGAG sequences can be achieved through either chemical synthesis or isolation and purification of active cell surface HLGAGs.

In the second theme, the present study provided the basis for:

1) Development of the HLGAG dry aerosol powder for clinical application. This would require a systematic study of dry powder formulation, dry powder inhaler (DPI), and patient respiratory biology. Dry powder formulation can be further improved in terms of respirability, dispersibility, and flowability. The design of DPI should take into consideration of the negative charges associated with polysaccharides.

2) Customization of pharmacokinetics of dry HLGAG powder. By incorporating certain excipient such as DPPC, lactose or other biodegradable polymers, it is likely to develop dry powder formulation with desired pharmacokinetic profiles for specific clinical applications. For instance, incorporation of biodegradable polymers can potentially prolong the half-life of the inhaled HLGAGs, minimize the dosing frequency and improve patient compliance.
**Abbreviations**

2-OST  2-O sulfotransferase  
6-OST  6-O sulfotransferase  
ACT  Activated clotting time  
AT III  Antithrombin III  
AUC  Area under the curve  
CB-1  Calcium binding site 1  
CB-2  Calcium binding site 2  
CE  Capillary electrophoresis  
C_{max}  Peak concentration observed  
CHO cells  Chinese hamster ovary cells  
CS  Chondroitin sulfate  
DEPC  Diethyl pyrocarbonate  
DS  Dermatan sulfate  
EGF  Epithelial growth factor  
FDA  Food and Drug Administration  
FGF  Fibroblast growth factor  
FGFR  FGF receptor  
G or GlcUA  Glucuronic acid  
H  Hexosamine (e.g. glucosamine and galactosamine)  
HCII  Heparin cofactor II  
Hep I  Heparinase I  
Hep III  Heparinase III  
HLGAG  Heparin-like glycosaminoglycans  
HPLC  High pressure liquid chromatography  
HS  Heparan sulfate  
HSPG  Heparan sulfate proteoglycans  
I or IdoUA  Iduronic acid  
IU  International standard units for anti-factor Xa activity
$K_a$  Absorption constant  
$K_e$  Elimination constant  
$K_d$  Dissociation constant  
$K_m$  Michaelis-Menton constant  
$K_{cat}$  Catalytic constant  
LB  Luria broth (rich medium for bacterial cultures)  
LMWH  Low molecular weight heparin  
MALDI  Matrix assisted laser desorption ionization  
MAP  Mitogen activated pathway  
MRT  Mean residence time  
MW  Molecular weight  
SDS-PAGE  Sodium dodecyl sulfate poly-acrylamide gel electrophoresis  
t $^{1/2}$  half-life  
t $^{1/2a}$  absorption half-life  
$T_{max}$  The time when $C_{max}$ is observed  
U  Uronic acid  
UFH  Unfractionated heparin  
VEGF  Vascular endothelial growth factor  
WRK  Woodward’s reagent K
Bibliography


