Roles of Heparan Sulfate in Amyloid-β Pathology and Hypoxia

ELINA HJERTSTRÖM

Heparan sulfate (HS) is a highly sulfated polysaccharide expressed on the cell surface and in the extracellular matrix, interacting with a large number of proteins. HS is implicated in human diseases, including different types of cancer and amyloid diseases such as Alzheimer's disease (AD). The aims of this thesis were to gain deeper insights into AD and cancer progression by elucidating the roles of HS in amyloid-β (Aβ) pathology and hypoxia.

The toxic Aβ-peptide is a key molecule in AD due to its ability to aggregate and form amyloid plaques in the brains of diseased patients. It has been reported that HS accumulates with Aβ in these amyloid plaques. We have found that HS is differentially accumulated with Aβ species within the amyloid plaques in the brains of AD patients. We also identified that the HS in the plaques originated from glial cells. Further, we investigated the role of HS in Aβ toxicity using cell models that either lack HS or express abnormal HS. The results show that cell surface HS mediates Aβ internalization and cytotoxicity.

Upregulation of heparanase, an endo-glucuronidase that specifically cleaves HS chains, in human cancers increases the potential of tumor cells to metastasize. Spalax, an animal model for hypoxic tolerance, expresses high levels of heparanase. Analysis of HS from different Spalax organs revealed a high sulfation degree and an atypical domain structure, likely modulated by high heparanase expression in the organs. Cells cultured under hypoxic conditions showed a similar HS domain structure and had an increase in heparanase mRNA. We propose that hypoxia-induced heparanase expression is relevant for tumor progression, a process often associated with oxygen deficiency.

Altogether, the findings in this thesis are important for future development of therapeutics aiming at interfering with HS functions in AD and cancer.

Keywords: Heparan sulfate, Alzheimer's disease, Aβ, Proteoglycan, Heparanase, Amyloid, Hypoxia, Cancer, Spalax

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


*These authors contributed equally to the work.

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<th>Full Form</th>
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<tbody>
<tr>
<td>Aβ</td>
<td>Amyloid-β</td>
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<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
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<td>AICD</td>
<td>APP intracellular domain</td>
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<td>APP</td>
<td>Amyloid precursor protein</td>
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<tr>
<td>BACE1</td>
<td>β-site APP-cleaving enzyme</td>
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<tr>
<td>CS</td>
<td>Chondroitin sulfate</td>
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<tr>
<td>CTF</td>
<td>C-terminal fragment</td>
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<tr>
<td>DS</td>
<td>Dermatan sulfate</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
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<td>EXT</td>
<td>Exostosin</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<td>GAG</td>
<td>Glycosaminoglycan</td>
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<tr>
<td>GlcA</td>
<td>Glucuronic acid</td>
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<tr>
<td>GlcN</td>
<td>Glucosamine</td>
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<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
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<td>GlcNS</td>
<td>N-sulfated glucosamine</td>
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<td>HIF</td>
<td>Hypoxia-inducible factor</td>
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<td>HS</td>
<td>Heparan sulfate</td>
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<td>HSPGs</td>
<td>Heparan sulfate proteoglycans</td>
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<td>IdoA</td>
<td>Iduronic acid</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>NA</td>
<td>N-acetylated</td>
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<tr>
<td>NDST</td>
<td>N-deacetylase/N-sulfotransferase</td>
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<td>NS</td>
<td>N-sulfated</td>
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<td>PG</td>
<td>Proteoglycan</td>
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<td>PS</td>
<td>Presenilin</td>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
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<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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Introduction

Heparan sulfate (HS), found at cell surfaces and in the extracellular matrix (ECM), is highly anionic due to the presence of sulfate groups along the saccharide chain. The HS-binding proteins include morphogens, cytokines, growth factors and receptors, which are crucial for fundamental biological processes such as development and homeostasis. In addition, HS is implicated in several pathological conditions including inflammation, infection, cancer and amyloid diseases such as Alzheimer’s disease (AD). The aims of this thesis were to gain deeper insights into AD and cancer progression by elucidating the roles of HS in Aβ pathology and hypoxia.

The first part of the thesis concerns the role of HS in amyloid-β (Aβ) pathology. The toxic Aβ peptide has a central role in AD where it aggregates and forms the characteristic amyloid plaques present in AD brain, of which HS is an established component. However, the role of HS in these amyloid deposits and how the Aβ peptide exerts its toxic effects are not fully understood.

The last part of this thesis focuses on the role of HS in hypoxia and its implications for tumor progression. Heparanase is an endo-glucuronidase that specifically cleaves HS chains. Although heparanase activity strongly correlates with the metastatic potential of tumor cells, there is limited knowledge regarding its regulation during cancer progression.
Background

Heparan sulfate proteoglycans

Nomenclature
Proteoglycans (PGs) are a family of proteins that carry covalently bound, unbranched glycosaminoglycan (GAG) chains. In heparan sulfate proteoglycans (HSPGs) the GAG chains are of heparan sulfate (HS)/heparin type. In other PGs the GAG chains can be chondroitin sulfate (CS)/dermatan sulfate (DS) or keratan sulfate. The GAG chain is composed of repeating disaccharide units where an amino sugar alternates with a uronic acid or galactose residue. HS/heparin is composed of glucosamine (GlcN) residues that can be $N$-acetylated (GlcNAc), $N$-sulfated (GlcNS) or in rare cases $N$-unsubstituted and glucuronic acid (GlcA) or iduronic acid (IdoA). CS/DS contains $N$-acetylgalactosamine and GlcA or IdoA whereas keratan sulfate is composed of GlcNAc and galactose residues. All of these GAGs contain sulfate substituents in various positions (Lindahl and Kjellen 1991).

Heparan sulfate biosynthesis
One of the main functions of HS is to bind to a wide array of proteins and thereby affect their functions. The binding depends on the presence of sulfate groups along the saccharide chain, which make the HS structure highly anionic. The complex and heterogenic structure of HS is generated through an elaborate biosynthetic process (Figure 1). The identification of tissue-specific structures of HS by structural analysis and specific antibodies against different HS-epitopes indicates that HS synthesis is well regulated (Maccarana et al. 1996; van Kuppevelt et al. 1998; Ledin et al. 2004; Kurup et al. 2007). Studies of mice and other model organisms emphasize the importance of HS biosynthetic enzymes for animal development and disease (Bulow and Hobert 2006).
Figure 1. The major steps in HS biosynthesis, polymerization and modifications (epimerization, N- and O-sulfation). The structures to the left represent examples of disaccharide structures from the different steps. The final structure can be divided into highly N-sulfated domains (NS), alternating N-acetylated/N-sulfated domains (NA/NS) and N-acetylated domains (NA).

The HS chain is synthesized in the Golgi apparatus on a primer tetrasaccharide sequence that starts with a xylose residue linked to a serine residue in the HSPG core protein, followed by two galactose residues and one GlcA residue. Galactosyltransferase I catalyzes the formation of the β₁,₄ glycosidic bond between the galactose and xylose residues. Defects in this enzyme are linked to progeroid Ehlers-Danlos syndrome that is characterized by mental retardation and defects in the connective tissue (Quentin et al. 1990). The addition of GlcNAc by EXTL2/EXTL3 (belonging to the exostosin enzymes (EXT)) determines that the GAG chain will be of HS/heparin type (Kim et al. 2001; Busse et al. 2007). A heterodimeric complex of EXT1 and EXT2 then adds alternating GlcA and GlcNAc residues (McCormick et al. 2000). Mutations in the EXT1 and EXT2 genes are associated with hereditary multiple exostoses; a dominantly inherited bone disorder (Cook et al. 1993; Wu et al. 1994).

The HS chain subsequently undergoes further modifications catalyzed by specific enzymes utilizing the sulfate donor PAPS. HS is N-deacetylated and N-sulfated by the NDST (N-deacetylase/N-sulfotransferase) enzymes that convert GlcNAc into GlcNS residues. PAPS may have a regulatory effect on NDST action, in addition to providing one of the substrates, since access to PAPS generates extended N-sulfated domains that are formed in a processive manner (Carlsson et al. 2008). Vertebrates have four NDSTs; NDST1 and NDST2 are expressed in most tissues while NDST3 and NDST4 are expressed during development and in the adult brain (Aikawa et al. 2001). Mice deficient in NDST1 die neonatally due to respiratory failure while mice
deficient in NDST2 have severe defects in mast cells due to the abnormal structure of heparin (Forsberg et al. 1999; Ringvall et al. 2000).

C5-epimerase is the enzyme that catalyzes the conversion of GlcA into IdoA. Epimerase knockout in mice results in a lethal phenotype with renal agenesis, lung defects and skeletal malformations (Li et al. 2003). 2-O-sulfotransferase is responsible for 2-O-sulfation of IdoA and GlcA, although with preference for IdoA (Rong et al. 2001). Knockout of 2-O-sulfotransferase in mice shows similar defects as the epimerase knockout mice (Bullock et al. 1998).

6-O- and 3-O-sulfation of glucosamine residues are catalyzed by specific 6-O-sulfotranferases (three isoforms and one alternatively spliced form) and 3-O-sulfotransferases (six isoforms). Deficiency in 6-O-sulfotransferase-1 leads to defective HS biosynthesis and late embryonic/perinatal lethality (Habuchi et al. 2007). 3-O-Sulfation of heparin/HS is crucial for the synthesis of sequences that specifically interact with the anticoagulant protein antithrombin (Lindahl et al. 1980). Unexpectedly, 3-O-sulfotransferase-1 knockout mice did not exhibit any procoagulant phenotype (HajMohammadi et al. 2003). The synthesized chain can undergo further modification at the cell surface and in the ECM by two 6-O-endosulfatases, which remove 6-O-sulfate groups, preferentially from trisulfated disaccharides in the HS chain (Ai et al. 2006).

The final HS product is composed of three different types of domains defined by their degree of sulfation. N-sulfated domains (NS) are the most modified sequences with the highest sulfation degree, N-acetylated domains (NA) are unmodified whereas N-acetylated/N-sulfated domains (NA/NS) are a mixture of the two (Figure 1) (Maccarana et al. 1996).

Core proteins
Depending on the type of core protein that carries HS chains, HSPGs are present on the cell surface, in the ECM or intracellularly. The cell membrane-bound HSPGs are the syndecans (four members) and the glypicans (six members) (Lindahl and Li 2009). Perlecan, agrin, and collagen XVIII are extracellular PGs with functions, in particular, in basement membranes (Iozzo et al. 2009). The only intracellular HSPG is serglycin (Kolset and Tveit 2008).

Syndecans
The transmembrane syndecans consist of a large ectodomain to which GAG chains are attached, a transmembrane region and a short cytoplasmic tail. There are usually at least three HS chains attached close to the N-terminus (Kirk-Safran et al. 2009; Multhaupt et al. 2009). The expression of the different syndecan members is cell- and tissue-specific and varies during development (Kim et al. 1994). They are generally described as co-receptors
that have key roles in signal transduction and can for instance act as co-receptors for fibroblast growth factors (FGFs) and transforming growth factor-beta (TGF-β) (Coutts and Gallagher 1995; Chen et al. 2004). Syndecans regulate integrin activity in different cell models, and integrins and syndecans are believed to provide a physical link between the ECM and the cytoskeleton (McQuade et al. 2006; Morgan et al. 2007). Clustering of syndecans and phosphorylation of the cytoplasmic tail are proposed to be important for recruiting cytoplasmic cytoskeleton and signaling molecules (Yoneda and Couchman 2003). Shedding of syndecan ectodomains by matrix metalloproteinases (MMPs) regulates signal transduction and adhesion (Manon-Jensen et al. 2010).

Syndecan-1, present on stromal and epithelial cells, carries both CS and HS chains (Kirn-Safran et al. 2009). Syndecan-1 knockout mice have no major developmental abnormalities. However, both knockout and overexpression of syndecan-1 lead to defect wound healing (Stepp et al. 2002; Elenius et al. 2004).

Syndecan-2, found on fibroblasts, has three conserved sites for HS attachment (Kirn-Safran et al. 2009). This isoform is implicated in angiogenesis (Fears et al. 2006) and is found to be upregulated in human colon cancer (Ryu et al. 2009).

Syndecan-3 has a large ectodomain with six potential sites for GAG attachment (both HS and CS) and is expressed in the nervous system, the adrenal gland and the spleen (Kirn-Safran et al. 2009). Mice that lack syndecan-3 have impaired hippocampus-dependent memory and show resistance to obesity under high fat diet (Kaksonen et al. 2002; Strader et al. 2004).

Syndecan-4, with three HS attachment sites, is found in most tissues but seems to be less abundant (Kim et al. 1994; Kirn-Safran et al. 2009). Its importance in angiogenesis and wound healing was demonstrated in syndecan-4 null mice showing delayed wound repair and impaired angiogenesis (Echtermeyer et al. 2001). However, the most crucial role of syndecan-4 seems to be in the formation of focal adhesions (Woods and Couchman 1994).

**Glypicans**

Glypicans, attached to the cell membrane through a glycosylphosphatidylinositol anchor, are characterized by cysteine-rich globular ectodomains with two to four HS chains attached close to the plasma membrane. Glypicans can regulate signaling of Wnts, Hedgehogs, FGFs and bone morphogenic proteins, either promoting signaling by stabilizing the interaction between growth factor and receptor or inhibiting signaling by sequestering growth factors, thereby competing with the growth factor receptor (Fransson et al. 2004; Filmus et al. 2008).

Glypican-1 is expressed in the central nervous system during development and in many other tissues in the adult, while glypican-2 expression is
limited to axons and growth cones in the developing brain (Fransson et al. 2004). Patients with brachydactyly E (mental retardation and limb abnormalities) were found to lack glypican-1 (Syrrou et al. 2002). Glypican-3 is expressed widely in the embryo (Pellegrini et al. 1998). Mutations in the glypican-3 gene are linked to the Simpson-Golabi-Behmel syndrome that is characterized by pre- and postnatal overgrowth (Pilia et al. 1996). Glypican-3 deficient mice display many of the abnormalities associated with this human disease (Cano-Gauci et al. 1999). Glypican-4, -5 and -6 are expressed in various organs in the embryo with restricted expression in adults (Fransson et al. 2004).

**Perlecan**

The perlecan core protein carries up to four HS chains. The large size of perlecan makes it a perfect linker molecule between the ECM and cell surface receptors (Iozzo et al. 2009). Indeed, perlecan mediates FGF signaling and suppression of perlecan therefore reduces growth factor signaling leading to decreased tumor growth and angiogenesis (Aviezer et al. 1994; Sharma et al. 1998). The importance of perlecan in developmental angiogenesis in zebrafish has been reported (Zoeller et al. 2008). On the other hand, an anti-angiogenic fragment, endorepellin is derived from the C-terminus of perlecan (Mongiat et al. 2003). Null mutations in the perlecan gene are lethal and result in severe developmental brain defects and skeletal abnormalities (Arikawa-Hirasawa et al. 1999).

**Agrin**

Agrin is mainly expressed in the brain, lung and kidney (Gesemann et al. 1998) and is involved in clustering of acetylcholine receptors (Nitkin et al. 1987). Homozygous agrin-deficient mutant mice form defective neuromuscular synapses (Gautam et al. 1996).

**Collagen XVIII**

Expression of collagen XVIII is prominent in the vascular and epithelial basement membranes of human and mouse tissues. Studies in mice have shown that collagen XVIII is critical for normal blood vessel formation in the eye. This may explain the eye defects seen in patients with Knobloch syndrome (with loss-of-function mutations in collagen XVIII) (Fukai et al. 2002). Collagen XVIII is proposed to play a negative regulatory role in angiogenesis presumably due to release of its C-terminal anti-angiogenic fragment endostatin (O'Reilly et al. 1997).

**Serglycin**

Serglycin, the only known intracellular PG, is found in endothelial and hematopoietic cells and can bear heparin (highly sulfated HS species) as well as CS chains (Kolset and Tveit 2008). Many studies have focused on sergly-
cin found in mast cells where it is involved in granulopoiesis and protease storage (Henningsson et al. 2002; Abrink et al. 2004).

Interactions between heparan sulfate and proteins
As mentioned above, the negative charge of HS makes it prone to interact with various proteins. HS-binding proteins include enzymes, cytokines, morphogens, growth factors, matrix proteins, lipoproteins and disease associated proteins such as amyloid proteins (Lindahl and Li 2009).

Many studies of HS-protein interactions are performed with heparin, used in clinics worldwide as an anticoagulant. A specific pentasaccharide sequence in heparin, responsible for binding to the anticoagulant protein antithrombin, was identified 30 years ago (Lindahl et al. 1980). However, this type of specific HS-protein interaction is rare, rather HS domain organization and levels of sulfation appear the most important parameters for HS-protein interactions (Kreuger et al. 2006; Lindahl 2007).

HS is often described as a co-receptor for FGFs that is required for effective activation of the FGF receptor (Yayon et al. 1991; Rapraeger 1993). Downstream FGF signaling includes the Ras-mitogen-activated pathway kinase (MAPK) pathway where the extracellularly signal-regulated kinases (ERK1/2) are considered responsible for the mitogenic response (Dailey et al. 2005). Most studies have employed heparin oligosaccharides to study the interaction between polysaccharide, FGFs and FGF receptors. Two different crystallographic models have illustrated how this interaction occurs, one symmetric model that contains two heparin molecules stabilizing the complex (Schlessinger et al. 2000) and one asymmetric model with one heparin molecule that has a bridging function (Pellegrini et al. 2000). Highly sulfated HS chains appear important for efficient formation of ternary complexes and sustained downstream signaling (Jastrebova et al. 2006; Jastrebova et al. 2010). 2-O-Sulfated IdoA was recently shown to be critical for FGF2 mediated signaling which may explain the observed phenotype in the epimerase and the 2-O-sulfotransferase null mice. However, it is not clear if the poor response to FGF is directly due to lack of IdoA or indirectly due to disturbed domain organization along the HS chain (Bullock et al. 1998; Li et al. 2003; Jia et al. 2009).

The expression of the potent angiogenic vascular endothelial growth factor (VEGF) is induced by hypoxia, oncogenes and different cytokines. HSPGs can mediate angiogenesis through binding to VEGF (except VEGF121 that lacks the HS-binding domain) (Houck et al. 1992; Stringer 2006). Furthermore, HS polarizes and guides sprouting endothelial cells to initiate vascular branch formation (Ruhrberg et al. 2002). The HS structure, important for VEGF binding, includes NS domains divided by a NA/NS domain (Robinson et al. 2006).
Heparanase

Functions

Heparanase was first identified in mouse mastocytoma, as an endo-β-D-glucuronidase capable of fragmenting macromolecular heparin (Ogren and Lindahl 1975). Subsequently, an enzyme was identified that could similarly cleave also HS chains (Oldberg et al. 1980), and more recent work pointed to a single, common enzyme species (Gong et al. 2003).

Heparanase is expressed in all tissues with significantly varied levels. The cleavage of HS chains generates fragments of 10-15 sugar units (4–7 kDa) in size (Fux et al. 2009b). Heparanase is not essential for animal development (Zcharia et al. 2009) but the enzyme is involved in embryonic implantation and development, wound repair, HS turnover, tissue remodeling and hair growth (Zcharia et al. 2004; Zcharia et al. 2005a; Zcharia et al. 2005b; Escobar Galvis et al. 2007). On the other hand, heparanase cleavage of HS directly influences the many HS-dependent biological functions (Li and Vlodavsky 2009). Transgenic mice that overexpress heparanase appear normal, are fertile and have a normal life span (Zcharia et al. 2004). Nonetheless, these mice show several physiological changes such as reduced fat deposition (Karlsson-Lindahl, personal communication), accelerated hair growth, excess branching and duct-widening of the mammary glands associated with enhanced neovascularization and disruption of the epithelial basement membrane compared with control mice. Further studies revealed that the mice have elevated wound angiogenesis (Zcharia et al. 2005b). The role of heparanase in angiogenesis is linked to ECM degradation and endothelial cell migration (vascular sprouting) as well as release of active FGF and VEGF from the ECM (Elkin et al. 2001). Furthermore, heparanase cleaves HS to release fragments that potentiate FGF receptor dimerization and formation of ternary complexes (Escobar Galvis et al. 2007; Nasser 2008). Heparanase also influences angiogenesis through a non-enzymatic mechanism, involving regulation of the VEGF gene (Zetser et al. 2006). The impact of heparanase function was further demonstrated in heparanase knockout mice (Zcharia et al. 2009). These mice do not show obvious phenotypes, are fertile and have a normal lifespan but show elevated expressions of MMP-2, -9 and -14. Partially overlapping functions of MMPs and heparanase may explain the mild phenotype in the mice.

Only one gene (HPSE) encodes for a protein with heparanase activity. A second gene was found coding for heparanase-2, a protein that shares 40% sequence identity with heparanase (McKenzie et al. 2000). Interestingly, a recent report showed that heparanase-2 is devoid of enzymatic activity but has a high affinity for HS and heparin and thereby has potential to actually inhibit heparanase activity (Levy-Adam et al. 2010).
Molecular properties

Heparanase is synthesized as a 65 kDa pro-enzyme and has a predicted TIM-barrel fold. Two acidic residues, a proton donor at Glu 225 and a nucleophile at Glu 343 are involved in the catalytic activity of heparanase and the enzymatic pH optimum is between 5.0 and 6.0 (Hulett et al. 2000). Pre-pro-heparanase is targeted to the ER via its own signal peptide, proteolytic cleavage at the N-terminus cleaves off the signal peptide and the 65-kDa latent heparanase is then shuttled to the Golgi. Heparanase is secreted via vesicles that bud off from the Golgi and interacts with cell surface HSPGs. Two HS binding domains have been identified in heparanase; Lys 158-Asn 162 and Gln 270-Lys 280 (Levy-Adam et al. 2005). The heparanase-HSPG complex is rapidly endocytosed and ends up in the lysosomes where the proteolytic processing occurs. A 6 kDa linker segment is cleaved off by cathepsin-L and the heterodimeric, active form of heparanase that consists of a 50 kDa and an 8 kDa subunit, participates in HS degradation (Levy-Adam et al. 2003; McKenzie et al. 2003; Gingis-Velitski et al. 2004b; Zetser et al. 2004; Abboud-Jarrous et al. 2008). Lipoprotein receptor-related protein and mannose 6-phosphate receptors can also mediate binding to the secreted heparanase precursor and convey it to the intracellular sites for processing and activation (Vreys et al. 2005). These high affinity receptors are used at low heparanase concentrations, while the low affinity HSPGs receptors are important at higher heparanase concentrations (Ben-Zaken et al. 2008). The active heparanase is stably stored in the lysosomal compartment, which may be a way to regulate its secretion and extracellular activities (Goldshmidt et al. 2002). Indeed, active heparanase can be secreted after stimulation with physiological concentrations of ADP and ATP (Shafat et al. 2006). The finding that heparanase can be secreted in response to a stimulus indicates that the extracellular levels of heparanase are tightly regulated. Furthermore, the gene expression of heparanase can be regulated by early growth response transcription factor, p53 and inflammatory cytokines (Li and Vlodavsky 2009). A newly identified COOH-terminal domain in heparanase seems to play roles in heparanase secretion and activity but also in facilitating Akt-phosphorylation, cell proliferation and tumor xenograft progression (Fux et al. 2009a).

Substrate specificity

Heparanase cleaves the glycosidic bond between a GlcA and a GlcNS in HS/heparin chains. Several groups have attempted to identify the optimal substrate for heparanase cleavage. A detailed study of heparanase substrate specificity used variously modified capsular polysaccharide generated from *Escherichia coli*, K5, as target (Pikas et al. 1998). The unmodified backbone that consists of GlcA and GlcNAc residues was not cleavable. Similarly resistant was a product obtained after modification of the backbone by *N*-deacetylation/*N*-sulfation and partial C5-epimerization of GlcA to IdoA.
Instead, structures that remained N-acetylated but were chemically (presumably randomly) O-sulfated were susceptible to heparanase cleavage. A 2-O-sulfated hexuronic acid, two monosaccharide units away from the cleavage site towards the reducing end seemed to be crucial for heparanase recognition (Figure 2). Other investigators, using purified recombinant heparanase and structurally defined oligosaccharides isolated from porcine intestine or bovine kidney, found that highly sulfated structures located on both sides of the actual cleavage site are important (Okada et al. 2002) (Figure 2). Recently, heparanase specificity was studied using purified recombinant heparanase and polysaccharide substrates that were modified by recombinant HS biosynthetic enzymes (Peterson and Liu 2010). These investigators suggested that heparanase cleaves the linkage between GlcA and a 3-O- or 6-O-sulfated GlcNS. Cleavage was claimed to be precluded when the hexuronic acid unit toward the reducing end from the cleavage site was a sulfated IdoA. However, our recent investigation shows that a HS substrate lacking IdoA residues is a significantly poorer substrate than a HS substrate with GlcA residues (Li et al, unpublished data).

![Proposed substrate sequence for heparanase cleavage](image-url)

*Figure 2. Proposed substrate sequence for heparanase cleavage (Pikas et al. 1998; Okada et al. 2002; Gong et al. 2003).*
Heparan sulfate in pathological conditions

Inflammation

HS modulates several steps in the inflammatory process. Close to the inflammatory site, P-selectins and E-selectins are expressed by endothelial cells. Glycosylated ligands on leukocytes bind weakly to the selectins and the leukocytes initiate their rolling along the vessel wall. HS in the vessel wall can interact with L-selectin on the rolling leukocytes and facilitate the rolling process. In the activation phase, chemokines are presented, bound to endothelial HS and induce leukocyte integrins to bind adhesion molecules on endothelial cells, which results in leukocyte arrest and transmigration through the endothelial cell layer (Ley et al. 2007). Heparin acts as an anti-inflammatory agent in asthma, ulcerative colitis and burns probably by competitive binding to chemokines (Young 2008) as well as P- and L-selectins (Wang et al. 2002). Indeed, HS interacts with several chemokines and cytokines including macrophage inflammatory protein (MIP)-1α, RANTES, interleukin (IL)-2, IL-8 and IL-10 (Najjam et al. 1998; Spillmann et al. 1998; Salek-Ardakani et al. 2000; Vives et al. 2002; Stringer et al. 2003). Our recent study demonstrates that an intravascular MIP-2 chemokine gradient on endothelial HS directs crawling of leukocytes toward transmigration sites (Massena et al. 2010).

Heparanase is upregulated in several inflammatory conditions and may potentially affect inflammatory reactions in different ways. HS degradation by heparanase can affect the architecture of the ECM and thereby facilitate migration of inflammatory cells (Li and Vlodavsky 2009). Furthermore, increased heparanase activity results in shorter HS chains (Escobar Galvis et al. 2007). Fragmented endothelial HS may have impaired ability to interact with chemokines and L-selectin, which would result in a reduced inflammatory response.

Amyloid diseases

HSPGs are present in all kinds of amyloid deposits, regardless of the type of amyloidogenic protein that is present (van Horssen et al. 2003). HSPGs co-localize with amyloid in the prion diseases, including Creutzfeldt-Jakob disease, Gerstmann-Straussler syndrome and scrapie (Snow et al. 1990). HSPGs are also present within islet amyloid deposits in type 2 diabetes mellitus (Young et al. 1992). HS binds to the N-terminal of the pro-islet amyloid polypeptide and stimulates amyloid formation (Abedini et al. 2006). Heparanase overexpression makes mice resistant to induced AA-amyloidosis, which suggests that the HS chains are involved in AA amyloid deposition (Li et al. 2005).
Alzheimer’s disease

The amyloid disorder AD is the most common form of dementia in the aging population. The patients suffer from cognitive deterioration, memory loss and behavioral changes. Senile plaques, neurofibrillary tangles (accumulation of hyperphosphorylated tau), cerebral amyloid angiopathy and inflammatory reactions are pathological characteristics of AD. Aβ is the major protein component of the senile plaques and has a central role in the pathology of AD. The known mutations in the gene coding for the amyloid precursor protein (APP) increase the production and/or deposition of Aβ in the brain, also seen in patients with sporadic AD. Current treatments for AD may relieve symptoms but do not alter the course of the disease. Attempts to delay disease progress include measures aiming at reducing Aβ production, inhibiting Aβ aggregation, enhancing Aβ-clearance but also immunotherapy (Selkoe 2001; Citron 2010; Crews and Masliah 2010).

Generation of Aβ

Aβ is formed by proteolytic cleavage of APP, a type 1 integral membrane glycoprotein expressed in all human tissues and is localized to the plasma membrane, ER, Golgi apparatus and mitochondria. APP is suggested to be involved in cell adhesion, synaptic function and neural plasticity, although its exact biological functions are unclear. Alternative splicing gives rise to several isoforms, of which the APP695 isoform is mainly expressed in neurons (Young and Bennett 2010). The human APP gene is located on chromosome 21 and over 20 mutations in this gene have been implicated with the hereditary form of familial AD and the related disease hereditary cerebral amyloid angiopathy. All these mutations are amino acid substitutions within or close to the Aβ domain (Brouwers et al. 2008).

The processing of APP that generates amyloid plaques can be referred to as the amyloidogenic pathway and requires release of the Aβ domain from APP by the β- and γ-secretases (Figure 3A). BACE1 (β-site APP-cleaving enzyme or β-secretase) is a transmembrane aspartyl protease. It cleaves APP giving rise to a soluble fragment called APPsβ and a remaining C-terminal fragment (β-CTF, C99). β-CTF is further cleaved by γ-secretase to yield Aβ peptides that range from 38 to 43 amino acids and an APP intracellular domain (AICD). γ-Secretase is a large protein complex comprised of presenilins-1 and -2 (PS-1 and PS-2), nicastrin, presenilin enhancer 2 and anterior pharynx-defective phenotype-1. PS-1 knockout mice have reduced Aβ levels and present altered skeletal and brain development. In humans, mutations in PS-1 are responsible for over 50% of familial AD cases. Mutations in PS-1 and PS-2 lead to increased Aβ42 production over Aβ40, possibly by inducing conformational changes in the γ-secretase complex. The complex is also responsible for processing Notch. Non-specific γ-secretase inhibitors inter-
fere with Notch cleaving and signaling and affect the gastrointestinal tract, thymus and spleen.

An alternative way to process APP is the non-amyloidogenic pathway (Figure 3A), which involves \( \alpha \)-secretase (also called ADAM9, ADAM10, ADAM17/TACE) cleavage of APP at Leu 17. This cleavage releases a soluble APP\(\alpha \) and leaves a remaining peptide (\(\alpha\)-CTF, C83) that is further processed by \(\gamma\)-secretase to yield a p3 fragment and AICD (Thinakaran and Koo 2008; Young and Bennett 2010).

A strongly confirmed genetic risk factor for early and late-onset AD is the \( \varepsilon \)4 allele of apolipoprotein E. This molecule functions as a ligand in receptor-mediated endocytosis of lipoprotein particles in normal physiology whereas in AD it is believed to affect A\(\beta\) aggregation and clearance (Kim et al. 2009).

Most of the transgenic mouse models used for investigation of AD are based on the familial AD mutations and overexpress APP, PS-1 or PS-2 alone or together. The Tg2576 mouse model of AD, carrying the Swedish double mutation K670N/M671L in APP, develops plaques as well as vascular deposits at 9-12 months of age. If a mutation in the PS-1 gene is introduced as well, the plaques develop as early as at four months of age (Crews and Masliah 2010).

**Figure 3.** Schematic illustration of APP processing. (A) APP processing via the non-amyloidogenic pathway (\(\alpha\)- and \(\gamma\)-secretases) and the amyloidogenic pathway (\(\beta\)- and \(\gamma\)-secretases). (B) Sequence of A\(\beta\), enlarged letters highlight the HHQK domain that interacts with HS.
**Aβ toxicity**

Aβ can spontaneously aggregate into β-sheet-rich fibrils *in vitro* which are similar to the aggregates found in amyloid plaques. These fibrils were long believed to cause neurodegeneration in AD but emerging data suggest that prefibrillar, soluble forms of Aβ are the key components in AD pathogenesis (Caughey and Lansbury 2003; Sakono and Zako 2010). This is supported by data showing that the number of plaques in a particular region of the brain does not correlate with neuron death, synaptic loss or cognitive impairment (Terry 2000). In addition, soluble Aβ oligomers are cytotoxic, cause synaptic loss, impaire cognitive functions, destabilize neuronal networks and inhibit long-term potentiation (Selkoe 2008; Palop and Mucke 2010; Sakono and Zako 2010). The mechanisms underlying the toxic effects are not clear but possible scenarios include formation of membrane pores, mitochondrial and lysosomal dysfunction as well as signaling pathway failure (Creus and Masliah 2010).

**Inflammation in Alzheimer’s disease**

The knowledge regarding the contribution of inflammation to AD pathogenesis is limited. It is known that activated microglia associate with senile plaques and produce pro-inflammatory cytokines such as IL-1 and tumor necrosis factor-α. According to the original view, this inflammatory reaction was neurotoxic and believed to accelerate the disease. However, it is now suggested that the inflammatory response actually can be beneficial and hence promote neuronal survival. The role of circulating monocytes infiltrating the brain in the immune reaction is currently being investigated (Cameron and Landreth 2009; Schwartz and Shechter 2010).

**Involvement of heparan sulfate in Alzheimer’s disease**

More than 20 years ago, HS was found deposited in amyloid plaques of AD (Snow et al. 1988). Direct interaction between Aβ and HS was demonstrated to involve a motif in Aβ, HHQK (Figure 3B) (Giulian et al. 1998). The interaction between Aβ and HS also affects the aggregation state of the peptide, the sulfate moieties promoting fibril formation (Castillo et al. 1999; McLaurin et al. 1999). *In vivo* and *in vitro* data have shown that heparin can attenuate Aβ toxicity in cell cultures as well as reduce plaque burden in a mouse model of AD (Bergamaschini et al. 2002; Bergamaschini et al. 2004). HS, CS and DS as well as a number of sulfated compounds and dyes have been demonstrated to attenuate Aβ toxicity in cell cultures (Pollack et al. 1995; Woods et al. 1995). In addition, glypican has been suggested to mediate Aβ toxicity in PC12 cells (Schulz et al. 1998). There are divergent results regarding the effect of HS/heparins on BACE1 activity. HS was shown to inhibit BACE1 activity in neuroblastoma cells (Scholefield et al. 2003) while
other groups reported that heparin activates BACE1 (Leveugle et al. 1997; Beckman et al. 2006).

Perlecan, agrin, glypicans, syndecans and collagen XVIII have all been associated with senile plaques. Agrin and glypican-1 are found in both non-fibrillar and fibrillar senile plaque, whereas the syndecans are only associated with fibrillar senile plaques (Castillo et al. 1997; Cotman et al. 2000; van Horssen et al. 2002; van Horssen et al. 2003). Furthermore, HS is found associated with neurofibrillary tangles (Perry et al. 1991). Interestingly, a recent study reported that neurofibrillary tangles are present in the brains of patients with mucopolysaccharidosis IIIB (an autosomal recessive disease where HS is accumulated in the lysosomes) (Ohmi et al. 2009).

Tumor progression and metastasis

HS, HSPGs and heparanase are widely associated with different types of cancers. HSPGs are crucial to the integrity of the basement membrane, proliferation of cells and angiogenesis, all key factors in tumor progression and metastasis (Li 2008).

Expression of heparan sulfate proteoglycans

Syndecan-1 is upregulated in different types of human cancers and the soluble ectodomain of syndecan-1 is present in the serum of several malignancies. Shed syndecan-1 is biologically active and can promote tumor growth and metastasis (Yang et al. 2002). Expression and shedding of syndecan-1 are proposed to be induced through heparanase-mediated stimulation of phosphorylated ERK, which in turn can activate MMP-9 (Mahtouk et al. 2007; Purushothaman et al. 2008). Both glypican-1 and -3 have been implicated in different cancers (Filmus and Selleck 2001). For example, glypican-1 is upregulated in breast and pancreatic cancer and presumably promotes growth factor signaling in cancer cells (Kleeff et al. 1998; Matsuda et al. 2001). Glypican-3 on the other hand is downregulated in breast and ovarian cancers while upregulated in hepatocellular carcinoma and neuroblastoma (Theocharis et al. 2010). Agrin is strongly associated with tumor angiogenesis in the liver (Ioizzo et al. 2009).

Heparanase activity

Increased heparanase expression in human tumors is linked to metastasis, tumor angiogenesis and reduced postoperative survival of cancer patients (Vlodavsky et al. 2008; Fux et al. 2009b). High heparanase expression in tumors may be ascribed to mutational inactivation of the tumor suppressor p53 during cancer development; this mutated form activates the heparanase promotor and hence heparanase expression (Baraz et al. 2006). Heparanase activity can induce angiogenesis by releasing growth factors such as FGF and VEGF upon heparanase cleavage (Vlodavsky et al. 1987; Folkman et al.
Lymphangiogenesis is stimulated by heparanase-induced expression of VEGF-C (Cohen-Kaplan et al. 2008). Furthermore, HS sulfation is increased as a consequence of heparanase overexpression, which promotes formation of ternary complexes with FGF1 or FGF2 and FGF receptor-1 (Escobar Galvis et al. 2007).

Heparanase promotes Akt-dependent endothelial cell invasion and upregulation of VEGF through a non-enzymatic mechanism involving Src activation (Gingis-Velitski et al. 2004a). Furthermore, a newly identified splice variant of heparanase, T5, devoid of enzymatic activity has protumorigenic properties (Barash et al. 2010).

**Hypoxia**

During tumor progression the interior of the tumor becomes hypoxic due to insufficient oxygen supply. Hypoxic conditions in tumors are associated with increased stability and activity of the hypoxia-inducible factor-1α (HIF-1α), a transcription factor inducing genes involved in cell invasiveness and angiogenesis. HIF consists of two sub-units, HIF-1α and HIF-1β; the HIF-1α subunit is degraded under normoxic conditions but stabilized under hypoxic conditions. This subunit binds to hypoxia responsive elements present on over 100 genes involved in erythropoiesis, angiogenesis, glucose metabolism and apoptosis and promotes their transcription (Ke and Costa 2006; Liao and Johnson 2007; Rademakers et al. 2008; Finger and Giaccia 2010). Few studies have evaluated the role of HS in hypoxia. Yet, HIF-1α is suggested to augment the FGF-2 response of endothelial cells by increasing the gene expression of HS biosynthetic enzymes (Li et al. 2002). Another example of hypoxia-induced change in HS structure is increased synthesis of antithrombin-binding sequences (Karlinsky et al. 1992). Furthermore, increase of perlecan and syndecan-2 expression in cells in response to hypoxia was recently reported (Asplund et al. 2009; Kaur et al. 2009). Endorepellin, the anti-angiogenic peptide derived from the C-terminus of perlecan, decreases tumor angiogenesis and increases tumor hypoxia (Bix et al. 2006). Hypoxia can also induce heparanase expression in adult rat hippocampus (Navarro et al. 2008).

It is worth mentioning that several of the HIF-1α induced genes such as VEGF, insulin growth factor 2, fibronectin, angiopepin-like 4 and platelet-derived growth factor-BB are also HS-binding proteins highly implicated in cancer (Bernfield et al. 1999; Cazes et al. 2006; Ke and Costa 2006; Abramsson et al. 2007; Semenza 2010).

**The Spalax animal model**

*Spalax*, a subterranean mole rat, has through natural selection developed physiological changes to deal with hypoxic stress. Since many of the changes are identical to those found in human tumors, this animal is an ex-
cellent mammalian model organism for studying hypoxia tolerance and tumor development. *Spalax* tolerates low oxygen levels for long periods of time; under laboratory conditions *Spalax* can survive at 3% oxygen for at least 11 hours without any deleterious effects or behavioral changes, while *Rattus* is near death after 2.5 hours (Avivi et al. 1999).

Many genes and proteins that are involved in increasing oxygen supply to tissues are preferentially expressed in *Spalax*. HIF-1α is expressed at high levels. *Erythropoietin* gene expression is regulated by HIF. Unlike *Rattus*, *Spalax* can respond to hypoxia by rapid and sustained expression of *Erythropoietin* presumably reflecting the need for erythrocyte production during drastic fall in oxygen supply (Shams et al. 2004). Already at the fetal stage, *Spalax* liver and kidney express higher levels of erythropoietin mRNA than *Rattus* indicating that fetal erythropoiesis is important for adaptation to hypoxic stress (Shams et al. 2005).

*Spalax* has a high expression of VEGF and an increased density of blood vessels although VEGF expression is not increased during hypoxic stress (Avivi et al. 2005b). This suggests that sufficient vascularization balances the effects of hypoxia. Similarly, the expression of the VEGF receptor 2 was higher in *Spalax* muscle than *Rattus* muscle under normoxia, but did not change after hypoxia was induced (Band et al. 2008).

The tumor suppressor gene p53 controls cellular responses to DNA damage and hypoxia. Upregulation of wild-type p53 during hypoxia results in apoptosis. p53 mutations are the most common genetic alteration in human cancers. Intriguingly, mutations in the *Spalax* p53 were reported to be identical to those found in human cancers. A mutation in the DNA binding domain in the *Spalax* p53 gene exchanges Arg for Lys at position 174, which appears to enhance transcription of cell cycle arrest and p53 stabilization/homeostasis genes, but reduce transcription of apoptosis genes (Ashur-Fabian et al. 2004; Avivi et al. 2005a; Avivi et al. 2007).

Heparanase is highly expressed in *Spalax* compared to its scarce expression in normal human tissues (Nasser et al. 2005). The heparanase splice variants that have been identified in *Spalax* may reflect the need for regulation of heparanase activity in response to hypoxic stress. One of the splice variants has a dominant negative effect on the wild-type enzyme and inhibits HS degradation, tumor growth and metastasis (Nasser et al. 2009) whereas another splice variant lacks enzymatic activity but enhances tumor growth (Nasser et al. 2005).

Heparan sulfate as a potential therapeutic target

Interfering with HS function is a chosen strategy for developing drugs aimed at treating cancer and amyloid diseases such as AD. Variants of heparin and HS mimetics are used for the purpose of binding HS ligands, thus competing with endogenous HS.
The low molecular-weight heparin Neuroparin penetrates the blood-brain barrier and prevents abnormal tau protein formation seen in AD (Dudas et al. 2002). The low molecular-weight heparin, Enoxaparin, attenuates neurotoxicity and proinflammatory activity of Aβ and is under evaluation in preclinical studies (Bergamaschini et al. 2009). However, the HS mimetic traminprosate (Alzhemed™) failed to show significant results in Phase III clinical trials (Sabbagh 2009). HS mimetics are efficient inhibitors of AA amyloidogenesis and have been reported to reduce the rate of progression of AA amyloid renal failure in clinical trials (Kisilevsky et al. 2007).

Low molecular weight heparins can prolong survival of cancer patients. The mechanisms behind this effect are not fully explored but are likely to include competition with endogenous HS for binding to various ligands involved in tumor progression and metastasis (Casu et al. 2008). Heparin reduces the number of lung metastases in rodents injected with melanoma, sarcoma, breast and colonic tumor cells (Mousa and Petersen 2009). Heparin can possibly mediate anti-metastatic effects by inhibiting P- and L-selectins, known to facilitate the interaction between tumor cells, platelets and leukocytes (Stevenson et al. 2007).

Inhibition of heparanase by blocking the active site and the heparin binding domains holds promise for inhibition of both angiogenesis and metastasis (Vlodavsky et al. 2007). To avoid the anticoagulant activities of heparin, N-acetylated and glycol-split heparins have been tested for inhibition of myeloma growth in mice with promising results (Naggi et al. 2005). These heparin variants effectively inhibit heparanase activity and fail to release ECM-bound growth factors, and thus are potent antiangiogenic and antime-tastatic agents. HS mimetics include the heparanase inhibitor PI-88, which inhibits both angiogenesis and metastasis and is currently in clinical trials. Other HS mimetics (tetra- and pentasaccharides) are under development and their ability to inhibit heparanase, bind to growth factors and affect blood coagulation is evaluated. Some of these compounds have shown potent antitumor effect in a mouse model of melanoma (Johnstone et al. 2010).
Present investigations

HS and the HS-degrading enzyme heparanase are implicated in many different pathological conditions. In AD pathology, HS plays roles in both amyloid deposition and Aβ toxicity. In cancer, HS and heparanase are involved in several of the steps that facilitate tumor progression and metastasis. The principal aims of this thesis were to gain deeper insights into AD and cancer progression by elucidating the roles of HS in Aβ pathology and hypoxia.

Aims of the study

- To investigate the roles of HS in Aβ deposition and Aβ toxicity (Papers I and II)
- To investigate the role of HS in hypoxia (Paper III)
Results and discussion

Heparan sulfate accumulation with Aβ deposits in Alzheimer’s disease and Tg2576 mice is contributed by glial cells (Paper I)

Amyloid plaques are frequently observed in AD and can be divided into two morphological categories, neuritic and diffuse (Selkoe 2001). Various types of HSPGs are expressed in both kinds of plaques (van Horssen et al. 2003) and HS is believed to catalyze fibrillization and facilitate early deposition of Aβ (McLaurin et al. 1999). It is suggested that diffuse plaques represent an early stage of amyloid deposition, which subsequently evolve into neuritic plaques with dense cores. Another possibility is that the different types of plaques form independently of each other (Armstrong 1998; Selkoe 2001).

To gain deeper understanding of the role of HSPGs in Aβ deposition we examined different types of plaques in cases of sporadic and familial AD and in a mouse model of AD (Tg2576) by immunohistochemistry.

Image analysis of sporadic AD revealed that HS had a 10-fold higher tendency to accumulate with Aβ40 neuritic plaques than Aβ42 diffuse plaques. In the case of Swedish APP mutation AD, we found that HS associated with all investigated Aβ40 neuritic plaques and occupied 60% of the plaque area. 30% of the Aβ42 diffuse plaques were positive for HS and occupied less than 5% of the plaque area. In both sporadic AD and Swedish APP mutation AD we found that the cores of the neuritic plaques associated with expression of glypican-1, syndecan-3 and GFAP (an astrocyte marker).

Tg2576, a mouse model of the Swedish mutation where the Aβ load is high, had a distinct plaque morphology, referred to as “doughnut-shaped” deposits. The plaque capsules stained positive for both Aβ40 and Aβ42, glypican-1 and syndecan-3. GFAP positive cells surrounded the plaques while CD11b-positive microglia cells were detected throughout the plaque body.

In the patient with PS-1 Δ9 AD, diffuse “cotton wool” plaques were found with almost exclusive staining for Aβ42. HS was associated with Aβ42 in 20% of the plaques but occupied only 3% of the total area of these plaques. We did not detect any expression of glypican-1, syndecan-3 or GFAP within these plaques.

Because glial cells were identified as the cellular source of HS, we wanted to evaluate the effect of Aβ exposure on HSPG expression. We isolated murine primary glial cells and stimulated them with Aβ. Both glypican-1 and syndecan-3 levels were increased upon Aβ exposure as demonstrated by Western blotting.

From these studies, we suggest that Aβ accumulation in amyloid deposits results in elevated levels of the cell surface HSPGs glypican-1 and syndecan-3 on glial cells. Associated HS chains accumulated, predominantly with
Aβ40, in neuritic plaques. The lack of a glial response in diffuse plaques explains the absence of HSPGs and the low HS content of these plaques. The diffuse plaques have been candidate precursors of neuritic plaques (Armstrong 1998). The absence of HS in diffuse plaques suggests that HS is not initiating plaque formation and implies that different types of plaques develop independently of each other. An alternative interpretation is that the shift from a diffuse plaque to a neuritic plaque depends on recruitment of glial cells with subsequent HSPG upregulation. Indeed, preliminary data from our heparanase overexpressing mouse imply that HS is important for mediating an inflammatory response to Aβ, whereas heparanase overexpression attenuates such response (Zhang et al, unpublished data). It is suggested that microglia participate in the formation of amyloid plaques by internalization of Aβ but also by driving fibrillogenesis within the plaque (Nagele et al. 2004). Although this needs further studies, HS expressed on glial cells is likely to participate in these processes.

Heparan sulfate mediates amyloid-beta internalization and cytotoxicity (Paper II)

Previous studies suggested that heparin and other GAGs may protect cells from Aβ toxicity (Pollack et al. 1995; Woods et al. 1995; Bergamaschini et al. 2002). In this study, we further investigated the role of HS in Aβ toxicity. We used the well-established cell line psgD-677 that lacks HS due to a mutated EXT1 gene (Lidholt et al. 1992; Wei et al. 2000). Aβ exposure induced cell death in wild-type cells, while the HS-deficient cells were resistant to the treatment. Moreover, only the wild-type cells were able to efficiently internalize the Aβ peptide. The pgsD-677 cells express three-fold higher levels of CS (Lidholt et al. 1992), suggesting that CS is not capable of mediating Aβ internalization and cytotoxicity. A recent study has demonstrated that plaque formation is initiated by cellular internalization of Aβ into multivesicular bodies, where it starts to form fibrils and eventually causes cell death (Friedrich et al. 2010). This internalization is dependent on endocytosis or phagocytosis, processes known to involve HS (Ji et al. 1993; Poon et al. 2010). Our data clearly demonstrate the essential role of HS in Aβ internalization.

We proceeded to test the importance of cell surface HS chains for Aβ interaction by using HEK293 cells that stably express heparanase and thus carry shorter HS chains. Incubation with Aβ had a modest toxic effect on heparanase overexpressing cells compared with HEK293 wild-type cells. This difference can possibly be explained by the release of HS chains with bound Aβ from the cell surface upon heparanase cleavage. Alternatively, the truncated HS chains generated by heparanase cleavage may bind weaker to Aβ than wild-type chains. Yet another possibility is that heparanase overex-
expression results in accelerated turnover of HSPGs, as shown for heparanase overexpressing mice (Escobar Galvis et al. 2007), which in turn would affect Aβ uptake.

The importance of cell surface HS in mediating Aβ uptake and toxicity was further illustrated in a third set of experiments using an endothelial cell line. Cell death was evaluated by flow cytometry after Aβ exposure in the presence or absence of heparin using annexin V and propidium iodide staining for apoptosis/necrosis. Added heparin clearly increased the amount of viable cells. Similar results were obtained if the cells were pretreated with heparin or if heparin was added together with Aβ. Furthermore, heparin prevented internalization of Aβ, presumably through inhibiting Aβ interaction with cell-surface HS. Notably, the chain length of heparin is crucial to increase cell survival; heparin oligomers of 6- and 12-mers were ineffective while 18-mers and full-length heparin (corresponding to ~50 sugar units) efficiently protected the cells. This size requirement is an important consideration in development of HS-based drugs aimed at ameliorating AD pathology. The protective effect of heparin on endothelial cells is potentially significant in view of the accumulation of Aβ in cerebral vessels that contributes to cerebral amyloid angiopathy (Thal et al. 2008).

In the above experiments, three different preparations of Aβ40 were used at various states of aggregation. The non-aggregated preparation (na-Aβ) and the aggregated preparation (a-Aβ) were toxic to the cells and were detected intracellularly. These preparations contain oligomeric, soluble forms of Aβ. The third preparation, agitated and aggregated Aβ (aa-Aβ) was toxic to the cells but could not be detected intracellularly. It is possible that small amounts of toxic oligomeric Aβ were present also in this preparation but at levels below the threshold for detection by immunocytochemistry. In agreement with our results, current notion favors the oligomeric species of Aβ as the toxic element (Sakono and Zako 2010).

Molecular structure of heparan sulfate from Spalax. Implications of heparanase and hypoxia (Paper III)

In this study, we analyzed HS from the subterranean Spalax mole rat. HS chain length, domain structure and disaccharide composition from the animal indicated a high activity of heparanase. Previous findings have demonstrated a high expression of heparanase in several Spalax tissues (Nasser et al. 2005). Our structural data verified that the high expression could be correlated with high enzymatic activity. Spalax has been extensively studied for its striking physiological adaptations to a hypoxic environment. We hypothesized that the high expression of heparanase could be a hypoxic adaptation, resulting for example in stimulated angiogenesis and release of growth factors bound to HS. Spalax HS was highly sulfated and shorter HS chains
were detected in the kidney and liver compared with the same tissues in mice. These observations are in line with previous findings of HS changes in heparanase overexpressing mice and cancer tissues (Escobar Galvis et al. 2007). To test if such changes could indeed be ascribed to hypoxia, we used HEK293 cells, mock-transfected and transfected with *Spalax* heparanase. The *Spalax* heparanase transfected cells showed extensively shorter chains than the mock-transfected cells and the HS domain structure was modulated toward patterns typically seen in HS from *Spalax* organs. Furthermore, phosphorylation of ERK was increased in the heparanase-transfected cells, indicating that high heparanase activity stimulates release of growth factors and subsequent down-stream signaling. Interestingly, HS from the mock cells cultured in hypoxic condition showed a HS domain structure that indicated increased heparanase activity and had upregulated heparanase mRNA. Our findings imply that increased heparanase expression and activity result in increased MAPK/ERK activity as a response to the hypoxic environment. Indeed, high heparanase activity has recently been reported to increase phosphorylation of ERK that in turn results in increased MMP-9 expression and syndecan shedding, implicated with invasion and angiogenesis of myeloma cells (Purushothaman et al. 2008; Purushothaman et al. 2010). Whether MMP expression and syndecan shedding are enhanced in *Spalax* remains to be elucidated.
Concluding remarks and future perspectives

HS is variously involved in physiological as well as pathological processes. Diseases such as multiple hereditary exostoses, Simpson-Golabi-Behmel syndrome, brachydactyly E and Knobloch syndrome are directly linked to mutations in HS biosynthetic enzymes or in HS core proteins. Further, HS affects inflammatory conditions, amyloid diseases and cancer, by interacting with key proteins, such as chemokines, amyloidogenic peptides and growth factors. The HS-cleaving enzyme heparanase also influences HS functions, for example by degrading basement membranes and releasing HS fragments with bound growth factors.

In AD, HS codeposits with the amyloidogenic peptide Aβ within amyloid plaques found in the brain of AD patients. We have further investigated the role of HS in these amyloid plaques and established that HS found in the plaques originates from glial cells (Paper I). This indicates that HS is involved in inflammation in AD, which is supported by our recent data showing that heparanase overexpression attenuates the inflammatory response to Aβ (Zhang et al, unpublished data). Signaling pathways such as the MAPK/ERK and TGF-β Smad2/3 pathways have previously been implicated in the inflammatory response in AD (Webster et al. 2006; Town et al. 2008). Whether changes in expression of HS and heparanase contribute to alterations in these pathways in AD remains to be elucidated.

HS and heparin have previously been implicated in Aβ cytotoxicity (Bergamaschini et al. 2002). We demonstrated that HS mediates internalization and cytotoxicity of Aβ (Paper II). Cells that completely lack HS failed to internalize the peptide and resisted Aβ toxicity. Moreover, truncated HS chains due to heparanase overexpression, or addition of heparin together with Aβ protected the cells from Aβ toxicity (Figure 4A). In order to elucidate if Aβ internalization and toxicity are dependent on specific HS structures, additional cell models that differ with regard to HS structure could be employed. Cell-surface HS might also play a role in phagocytosis by glial cells of Aβ and necrotic cells. Indeed, HS has previously been reported to be involved in phagocytosis of necrotic cells (Poon et al. 2010).

Upregulated expression of heparanase in tumor tissues results (at least in selected studied cases) in increased HS sulfation, which may facilitate formation of ternary complexes with growth factors and receptors (Escobar Galvis et al. 2007). This is in agreement with our study (Paper III) of an animal model for hypoxic tolerance, the Spalax mole rat, with a constitutive
high expression and activity of heparanase and associated highly sulfated HS. In fact, we showed that heparanase expression leads to increased MAPK/ERK activity, presumably a result of augmented cell-surface signaling. We demonstrated that heparanase expression is induced by hypoxia and hypothesize that a similar condition occurs during tumor progression, associated with oxygen deficiency. The frequent and high heparanase expression observed in human tumors may thus conceivably be ascribed to hypoxia. Subsequent increase in MAPK/ERK activity would promote angiogenesis and tumor growth (Figure 4B). Our study emphasizes the importance of developing heparanase inhibitors for anti-cancer therapy. Targeting of the heparanase C-domain would possibly affect several heparanase functions including secretion, activity, Akt phosphorylation, cell proliferation and tumor progression (Fux et al. 2009a).

Changes in HS structure and expression, as well as heparanase activity occur in different pathological processes without necessarily being the primary cause of the particular diseases. The changes are perhaps an attempt to maintain homeostasis when the fine balance of molecular events in organs is disturbed during disease. Earlier reports have implicated HS in both Aβ pathology and hypoxia and we have identified new important roles of HS in these conditions. More detailed studies are needed to understand the mechanisms by which HS contributes to different pathological conditions, for example, how changes in HS structure affect different signaling pathways. We showed that hypoxia can induce expression and activity of heparanase and as a result change HS structure. Identifying other factors that can regulate heparanase expression and activity is important for further understanding heparanase functions. The complex and well-regulated HS biosynthesis indicates that changes within this system have the potential to affect both biological and pathological processes; a deeper knowledge regarding regulation of HS biosynthesis is therefore needed.
Figure 4. Model of the different roles of HS in Aβ pathology and hypoxia. (A) Aβ interacts with cell surface HSPGs mediating Aβ internalization and toxicity. Cells that lack HS, overexpress heparanase, or are treated with heparin fail to internalize Aβ and are resistant to Aβ toxicity. (B) (1) Hypoxia in tumor cells induces the expression of heparanase, which is secreted, and acts on the HS chains of HSPGs (2) to release growth factors that through receptor interactions result in increased MAPK/ERK activity (3). MAPK/ERK activity can induce expression of MMP-9 and syndecan shedding and further stimulate tumor growth and angiogenesis (4).
Heparansulfat (HS) är en kolhydrat som återfinns på cellytan och utanför cellerna i kroppens alla vävnader. HS är starkt negativt laddat och binder till en mängd proteiner som är viktiga för grundläggande biologiska processer. I studier på möss har det visat sig att avsaknad av HS leder till att embryonalutvecklingen avstannar och embryot dör på ett mycket tidigt stadium. Dessutom verkar HS vara inblandat i flera olika sjukdomstillstånd såsom cancer och amyloida sjukdomar (sjukdomar där olika proteiner klumpar ihop sig och förstör organens funktion).

Alzheimers sjukdom är en amyloidsjukdom som karakteriseras av amyloida inlagringar i hjärnan. De amyloida inlagringarna består till stor del av molekylen amyloid-β (Aβ), som också orsakar celldöd. Vi har undersökt hur HS ansamlas i dessa amyloidinlagringar och hur HS påverkar Aβ-toxicitet. Vi har även studerat hur HS påverkas av låga syrenivåer, hypoxi, något som är vanligt i tumörer.

Studierna i denna avhandling har visat att HS finns uttryckt på immunceller kring amyloidinlagringarna hos Alzheimerspatienter och att HS som finns på cellernas yta möjlig gör för Aβ att ta sig in i cellerna och orsaka celldöd. Vi har dessutom visat att hypoxi leder till att enzymet heparanas, som bryter ner HS, ökar i mängd och aktivitet. Detta kan vara viktigt för att frigöra tillväxtfaktorer som behövs för bildning av nya blodkärl och därmed ökning av syrettillförseln.

Sammanfattningsvis så har studierna i denna avhandling gett nya inblickar i hur HS påverkar olika sjukdomsprocesser. Detta är viktig kunskap som behövs för att utveckla läkemedel mot olika sjukdomstillstånd där HS deltar.
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rywhere but they soon realized that heparan sulfate is the most fascinating molecule. I was placed in an office together with Christoffer Taaaaammmmmm, who I was supposed to marry?! He was constantly arguing with the crazy and fun Irmeli Barkefors, maybe she was allergic to him? When Anna Eriksson, a smart person and a fantastic friend, joined the corridor, my life was divided into the time before and after Anna. Anna started in the group of the helpful, skilled and sporty researcher Dorothe Spillmann, and the atmosphere in the new corridor was now very nice! Jimmy Larsson also contributed to the nice atmosphere even though not for that long (he didn’t have the time). Thanks Tobias Bergström, Inger Eriksson, Peder Fredlund Fuchs, Johan Heldin, Cecilia Annéren, Maud Forsberg, Xiao-qun Zhang, Ulrika Wallenquist, Karin Forsberg Nilsson, Sebastian Le Jan, Johan Kreuger, Ludwig Peterson, Pär Gerwins, Lars Lundin and Dagmar Sandbäck Pikas for contributing to the nice feeling in the lab! We were all very pleased to have the amyloid experts, Robert Kisleovsky and John Ancsin from Canada as well as the nice researchers, Bo Wang and Jian Wang from China visiting the lab. Starting from November 2009, I spent 10 months with my beloved son Valter and came back to the lab to write this thesis. This time I was put in the quiet-room shared with several nice persons; Katarina Holmborn Garpenstrand, Anders Dagälv, Beata Filipek Gorniok and Zsolt Kasza. New pleasant persons had arrived to our corridor; Audrey Deligny, Sara Pijuan Galitó, Ramesh Namburi, Sara Thorslund and Natalia Strid while Rashmi Ramachandra and Juan were away from the lab taking care of Lilly and Maanvi. During these years I have had excellent technical and administrative help from Erika Engström, Kerstin Lidholt, Rehné Åkerblom, Barbro Lowisin, Marianne Wigenius and Olav Nordli. I have shared many nice dinners and laughs with my dear friend Kerstin Ahlgren during the 10 years we have been studying in Uppsala. I have also shared many discussions about life with my warm and caring friend Jenny Sågetorp during these years.

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tes with the phenotype of the Simpson-Golabi-Behmel syndrome."


highly sulfated domains and can be liberated by K5 lyase." J Biol Chem 281(3): 1731-40.
Shams, I., A. Avivi and E. Nevo (2004). "Hypoxic stress tolerance of the blind subterranean mole rat: expression of erythropoietin and hypox-
oglycans in malignancy and their pharmacological targeting."

*FEBS J.*


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