Regulation, function and clinical significance of heparanase in cancer metastasis and angiogenesis

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Abstract

Heparanase is an endoglycosidase which cleaves heparan sulfate (HS) and hence participates in degradation and remodeling of the extracellular matrix (ECM). Heparanase is preferentially expressed in human tumors and its over-expression in tumor cells confers an invasive phenotype in experimental animals. The enzyme also releases angiogenic factors from the ECM and thereby induces an angiogenic response in vivo. Heparanase upregulation correlates with increased tumor vascularity and poor post-operative survival of cancer patients. Heparanase is synthesized as a 65 kDa inactive precursor that undergoes proteolytic cleavage, yielding 8 and 50 kDa protein subunits that heterodimerize to form an active enzyme. Human heparanase is localized primarily within late endosomes and lysosomes and occasionally on the cell surface and within the cell nucleus. Transcriptional activity of the heparanase promoter is stimulated by demethylation, early growth response 1 (EGR1) transcription factor, estrogen, inflammatory cytokines and inactivation of p53. N-acetylated glycol-split species of heparin as well as siRNA heparanase gene silencing inhibit tumor metastasis and angiogenesis in experimental models. These observations and the unexpected identification of a single functional heparanase, suggest that the enzyme is a promising target for anti-cancer and anti-inflammatory drug development. Heparanase exhibits also non-enzymatic activities, independent of its involvement in ECM degradation and changes in the extracellular microenvironment. For example, cell surface expression of heparanase elicits a firm cell adhesion, reflecting an involvement in cell–ECM interaction. Heparanase enhances Akt signaling and stimulates PI3K- and p38-dependent endothelial cell migration and invasion. It also promotes VEGF expression via the Src pathway. The enzyme may thus activate endothelial cells and elicits angiogenic and survival responses. Studies with heparanase over-expressing transgenic mice revealed that the enzyme functions in normal processes involving cell mobilization, HS turnover, tissue vascularization and remodeling. In this review, we summarize the current status of heparanase research, emphasizing molecular and cellular aspects of the enzyme, including its mode of processing and activation, control of heparanase gene expression, enzymatic and non-enzymatic functions, and causal involvement in cancer metastasis and angiogenesis. We also discuss clinical aspects and strategies for the development of heparanase inhibitors.

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Keywords: Heparanase; Heparin; Metastasis; Angiogenesis

Abbreviations: bFGF, basic fibroblast growth factor; DTH, delayed type hypersensitivity; ECM, extracellular matrix; HS, heparan sulfate; HSPGs, heparan sulfate proteoglycan; VEGF, vascular endothelial growth factor

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1. Introduction

Heparan sulfate proteoglycans (HSPGs) are ubiquitous macromolecules associated with the cell surface and ECM of a wide range of cells of vertebrate and invertebrate tissues (Iozzo & San Antonio, 2001; Kjellen & Lindahl, 1991). The basic HSPG structure consists of a protein core to which several linear heparan sulfate (HS) chains are covalently O-linked. The polysaccharide chains are typically constituted by repeating disaccharide units composed of a uronic acid (either D-glucuronic, GlcA or L-iduronic, IdoA) and D-glucosamine (either N-acetylated, GlcNAc or N-sulfated, GlcNSO₃) that are modified at various positions by sulfation, epimerization and N-acetylation, yielding clusters of sulfated disaccharides separated by low or non-sulfated regions (Iozzo & San Antonio, 2001; Kjellen & Lindahl, 1991). HS glycosaminoglycans bind to and assemble ECM proteins (i.e., laminin, fibronectin, collagen type IV) and thereby contribute significantly to the ECM’s self assembly and integrity. HS also play important roles in cell–cell and cell–ECM interactions. Moreover, the HS chains, unique in their ability to bind a multitude of proteins, ensure that a wide variety of bioactive molecules bind to the cell surface and ECM and thereby contribute in the control of normal and pathological processes, among which are morphogenesis, tissue repair, inflammation, vascularization and cancer metastasis (Bernfield et al., 1999; Iozzo & San Antonio, 2001; Kjellen & Lindahl, 1991). Apart from sequestration of bioactive molecules, transmembrane (syndecans) and phospholipid-anchored (glypicans) HSPGs have a co-receptor role in which the proteoglycan, in concert with the other cell surface molecules, comprises a functional receptor complex that binds the ligand and mediates its action (Bernfield et al., 1999; Iozzo & San Antonio, 2001; Kjellen & Lindahl, 1991). HS also tether a multitude of growth factors, chemokines, cytokines...
and enzymes to the ECM and cell surface, providing a low affinity storage depot (Bernfield et al., 1999; Folkman et al., 1988; Vlodavsky et al., 1987). Cleavage of HS side chains is therefore expected not only to alter the integrity of the ECM, but also to release HS-bound biological mediators. In addition, HS fragments are also capable of modulating the activity of growth factors such as bFGF and VEGF and enzymes such as thrombin and lipoprotein lipase (Vlodavsky, Bar-Shavit, Korner, & Fuks et al., 1993). Structural requirements of HS (i.e., sulfation level, chain length) for growth factor sequestration, release and stimulation, were reported (Miao, Ishai-Michaeli, Atzmon, Peretz, & Vlodavsky, 1996; Vlodavsky, Miao, Medalion, Danagher, & Ron, 1996). Altogether, heparanase may have global effects on growth factor signaling and bioavailability. The ECM provides an essential physical barrier between cells and tissues, as well as a scaffold for cell growth, migration, differentiation and survival, and undergoes continuous remodeling during development and in certain pathological conditions such as wound healing and cancer (Timpl & Brown, 1996). ECM-remodeling enzymes are thus expected to profoundly affect cell and tissue function. While intensive research focused on enzymes capable of degrading and remodeling protein components in the ECM (Stetler-Stevenson, Aznavoorian, & Liotta, 1993; Werb, 1997), less attention was paid to enzymes (e.g., heparanase) cleaving glycosaminoglycan side chains.

Heparanase is an endo-β-D-glucuronidase capable of cleaving HS side chains at a limited number of sites, yielding HS fragments of still appreciable size (∼5–7 kDa) (Freeman & Parish, 1998; Pikas, Li, Vlodavsky, & Lindahl, 1998; Vlodavsky & Goldshmidt, 2001). Heparanase activity has long been detected in a number of cell types and tissues. Importantly, heparanase activity correlated with the metastatic potential of tumor-derived cells, attributed to enhanced cell dissemination as a consequence of HS cleavage and remodeling of the ECM barrier (Parish, Freeman, & Hulett, 2001; Vlodavsky & Friedmann, 2001). Similarly, heparanase activity was implicated in neovascularization, inflammation and autoimmunity, involving migration of vascular endothelial cells and activated cells of the immune system (Dempsey, Brunn, & Platt, 2000; Parish et al., 2001; Vlodavsky & Friedmann, 2001). In spite of the attractive clinical relevance of the pro-metastatic, pro-inflammatory and pro-angiogenic activities of heparanase, progress in the field was slow, largely due to the lack of recombinant heparanase, molecular probes, antibodies and a simple bioassay to quantify heparanase activity.

Heparanase activity was attributed to proteins with molecular weight ranging from 8 to 130 kDa, raising the possible existence of several HS-degrading endo-glycosidic enzymes (Dempsey et al., 2000; Parish et al., 2001; Vlodavsky & Friedmann, 2001). This confusion was solved when the cloning of a single human heparanase cDNA sequence was independently reported by several groups (Hulett et al., 1999; Kussie et al., 1999; Toyoshima & Nakajima, 1999; Vlodavsky et al., 1999). Thus, unlike the large number of proteases that can degrade polypeptides in the ECM, one major heparanase (i.e., heparanase 1) appears to be used by cells to degrade the HS side chains of HSPGs. Heparanase 2, having 3 splice variants and showing ∼35% homology with heparanase 1, was cloned, although no enzymatic activity was demonstrated (McKenzie et al., 2000). Whereas heparanase 2 mRNA expression shows a wide distribution in normal tissues (McKenzie et al., 2000), expression of heparanase 1 is restricted primarily to the placenta, keratinocytes, platelets and activated cells of the immune system, with little or no expression in connective tissue cells and most normal epithelia (Parish et al., 2001; Vlodavsky & Friedmann, 2001). Up-regulated expression of heparanase 1 was noted in essentially all human tumors examined, inflammation, wound healing and diabetic nephropathy (Dempsey et al., 2000; Parish et al., 2001; Vlodavsky & Friedmann, 2001). During embryogenesis, the enzyme is preferentially expressed in cells of the developing vascular and nervous systems (Goldshmidt et al., 2001).

With the availability of appropriate reagents, heparanase research entered a new era. Recent studies have shown that heparanase is up regulated in an increasing number of primary human tumors (Simizu, Ishida, & Osada, 2004). Heparanase upregulation correlated with increased lymph node and distant metastasis, increased micro-vessel density and reduced post-operation survival of cancer patients (see below), providing a strong clinical support for the pro-metastatic and pro-angiogenic features of the enzyme. Here, we summarize recent progress in molecular and cellular aspects of heparanase, emphasizing its causal involvement in cancer metastasis and angiogenesis, and discuss strategies for the development of heparanase inhibitors. Notably, heparanase activity liberates short HS fragments that may exert multiple effects of their own. This aspect has been reviewed by others (Sanderson et al., 2005; Sanderson, Yang, Suva, & Kelly, 2004; Saisishekaran, Shrivar, Venkataraman, & Narayanasami, 2002) and is beyond the scope of this review.
2. Heparanase gene regulation

2.1. Methylation

Induced expression of heparanase in human cancer (see below), inflammation (Edovitsky et al., 2006; Parish et al., 2001; Vlodavsky et al., 1992), diabetic nephropathy (Levidiotis, Freeman, Punler et al., 2004; Levidiotis, Freeman, Tikellis, Cooper, & Power, 2004; Levidiotis, Freeman, Tikellis, Cooper, & Power, 2005; Levidiotis, Kanellis, Ierino, & Power, 2001), and other pathological conditions suggests a transcriptional regulation. In addition to multiple genetic alterations that govern cell transformation, epigenetic processes, marked by hypermethylation of the promoter region, contribute significantly to gene transcription and cancer progression, for example by down regulation of tumor suppressor and DNA repair genes. Several studies have convincingly shown that promoter methylation status plays an important role in heparanase gene transcription. By examining a series of tumor-derived cell lines, we have found that cells which exhibit heparanase activity also harbor at least one unmethylated allele (Shteper et al., 2003). In contrast, cell lines which exhibit no heparanase expression or activity, such as C6 rat glioma and JAR human choriocarcinoma, were found to harbor fully methylated alleles. Treating these cells with demethylating agents such as 5-azacytidine restored heparanase activity and was accompanied by augmented metastatic capacity in vivo (Shteper et al., 2003). This augmentation was suppressed in mice treated with laminar sulfate (Shteper et al., 2003), compound that inhibits heparanase activity and experimental metastasis (Miao et al., 1999), supporting the intimate involvement of heparanase activity in metastatic dissemination. Subsequent studies revealed a similar correlation with prostate and bladder cancer-derived cell lines, and, moreover, with prostate and bladder tissue. Thus, significantly higher promoter methylation was found in benign prostatic hyperplasia (BPH) and in normal bladder than in carcinomas, inversely correlating with heparanase expression (Ogishima, Shiina, Breault, Tabatabai et al., 2005; Ogishima, Shiina, Breault, Terashima et al., 2005). Interestingly, Ogishima et al. have noted a correlation between heparanase expression by bladder and prostate carcinomas and the expression levels of early growth response 1 (EGR1), a transcription factor implicated in heparanase gene transcription (de Mestre, Khachigian, Santiago, Staykova, & Hulett, 2003; de Mestre et al., 2005). In contrast with other transcription factors such as SP1 and Ets, associated with basal heparanase transcription levels (Jiang et al., 2002), EGR1 appears to be related to inducible transcription of the heparanase gene (de Mestre et al., 2003, 2005). EGR1 is rapidly induced in response to a variety of signals such as growth factors, cytokines and injury, is up regulated in human tumors (Abdulkadir et al., 2001) and is strongly implicated in tumor angiogenesis (Khachigian, 2004). The later function may be related, at least in part, to the strong pro-angiogenic capacity of heparanase.

2.2. Tumor suppressor p53

Little is known about physiologically relevant repressors of heparanase gene transcription. Applying chromatin immunoprecipitation (ChIP) analysis, we have demonstrated that wild type p53 inhibits transcription of the heparanase gene by direct binding to its promoter. Moreover, this inhibition involved recruitment of histone deacetylases (HDACs). On the other hand, mutated, tumor-derived variants of p53 loss this inhibitory ability and in some cases even up regulate heparanase gene expression (Baraz, Haupt, Elkin, Perez, & Vlodavsky, 2006). These results indicate that under normal conditions the heparanase gene is constitutively inhibited by wild type p53. Mutational inactivation of p53 during cancer development leads to transcriptional activation of heparanase expression, providing a possible molecular mechanism for the frequent increase in heparanase levels observed in the course of tumorigenesis.

2.3. Estrogen and breast cancer

While gene methylation plays a critical role in heparanase transcriptional regulation, additional regulatory mechanisms may operate, locally or systemically. An example for a systemic regulator is the hormone estrogen, one of the main driving forces in breast tumorigenesis. We have identified putative estrogen response elements in the heparanase promoter and demonstrated their functionality by a luciferase reporter gene driven by the heparanase promoter (Elkin et al., 2003). Physical association between estrogen receptor (ER) and the heparanase promoter was confirmed by ChIP analysis. Luciferase activity and heparanase mRNA levels were significantly increased in estrogen receptor-positive MCF7 human breast carcinoma cells, but not in estrogen receptor-negative human breast carcinoma cells upon treatment with estrogen, induction that could be prevented by the estrogen inhibitor ICI 182,780 (Elkin et al., 2003). Similarly, estrogen facilitated heparanase expression by MCF7 cells embedded in Matrigel and implanted sub cutaneously, resulting in plugs that are more vascularized, again supporting the pro-angiogenic
properties of the enzyme. These results indicate that heparanase expression induced in breast epithelium by estrogen, contributes, among other factors, to primary breast tumor growth and neovascularization. This effect could be particularly important at the initial stages of breast tumorigenesis, when more than 70% of all tumors are reportedly ER positive. While these observations are relevant to breast cancer, mechanisms that promote heparanase expression in tissues other than breast are currently poorly understood.

2.4. Inflammatory mediators

Polyanionic compounds which inhibit heparanase enzymatic activity (e.g., heparin) also inhibit inflammatory responses (Bartlett, Underwood, & Parish, 1995; Lider et al., 1989; Parish et al., 1998). This effect may be attributed to inhibition of heparanase produced by activated T lymphocytes, regarded as the primary cellular source of the enzyme in inflammation (Naparstek, Cohen, Fuks, & Vlodavsky, 1984; Vlodavsky et al., 1992). In order to elucidate the source and biological significance of heparanase in inflammation, we examined the role of heparanase in a delayed-type hypersensitivity (DTH) mouse model, widely used to assess cell mediated immunity characterized by a granulomatous response consisting of macrophages, monocytes and T lymphocytes. For this purpose, mice were sensitized on the abdominal skin with an antigen (i.e., oxazolone) and DTH sensitivity was elicited 4–5 days later by challenging the mice with the same antigen administered topically to each side of the ear (Edovitsky et al., 2006). Surprisingly, heparanase immunostaining revealed that endothelial cells are the primary source of the enzyme under this experimental setting (Edovitsky et al., 2006). Furthermore, TNFα and IFN-γ, key mediators of DTH inflammation, up regulated heparanase gene expression and enzymatic activity in cultured endothelial cells (Edovitsky et al., 2006) and T lymphocytes (Sotnikov et al., 2004), consistent with a recent report demonstrating induced heparanase expression and secretion by endothelial cells treated with TNFα (Chen et al., 2004). Computerized analysis of the heparanase gene 1.9-kb promoter sequence revealed two consensus interferon-stimulated response elements (ISREs) that specifically bind transcription factors activated by interferon (Edovitsky et al., 2006). These results point to the vascular endothelium as an important cellular source of heparanase enzymatic activity that, in turn, allows for remodeling of the vascular basement membrane, increased vessel permeability and extravasation of metastatic tumor cells, leukocytes and plasma proteins.

3. Molecular properties

3.1. Heparanase structure

The heparanase gene (~50 kb) is located on human chromosome 4q21.3 and is linked to the genetic marker D4S400 (Dong, Kukula, Toyoshima, & Nakajima, 2000). The gene is expressed as 5 and 1.7 kb mRNA species, generated by alternative splicing. The 5 kb form contains 14 exons and 13 introns, whereas in the short form the first and 14 exons have been spliced out. Only one gene (HPSE) has been shown to encode for a protein with heparanase activity (Hulett et al., 1999; Kussie et al., 1999; Toyoshima & Nakajima, 1999; Vlodavsky et al., 1999). Sequence analysis revealed that heparanase is highly conserved, with similar sequences found in human, rat, mouse, cow, chicken, mollusks and zebra fish (Goldshmidt et al., 2001; Parish et al., 2001). The gene has not been identified in drosophila and C. elegans. The human heparanase cDNA contains an open reading frame that encodes a polypeptide of 543 amino acids with a molecular weight of 61.2 kDa. The active heparanase purified from placenta, platelets and various cell lines was found to lack its N-terminal 156 amino acids, suggesting post-translational proteolysis of the heparanase polypeptide (Parish, Freeman, Brown, Francis, & Cowden, 1999; Toyoshima & Nakajima, 1999; Vlodavsky et al., 1999). In fact, active heparanase was subsequently reported to be a heterodimer consisting of a 50 kDa subunit (Lys158–Ile543) associated non-covalently with an 8 kDa peptide (Gln36–Glu109). The intervening 6 kDa peptide (Ser110–Gln157) is excised by proteolysis (Fairbanks et al., 1999; Levy-Adam, Miao, Heinrikson, Vlodavsky, & Ilan, 2003; McKenzie et al., 2003). Based on the predicted amino acid sequence, the 50 kDa subunit of human heparanase contains six putative N-glycosylation sites. Although glycosylation was not required for enzyme activity, secretion of heparanase was regulated by glycosylation (Simizu, Ishida, Wierzbza, & Osada, 2004). The sequence also contains a 35-amino acid N-terminal signal sequence (Met1–Ala35), and a C-terminal hydrophobic domain (Pro515–Ile534). Heparanase has been shown to be related to members of the clan A glycosyl hydrolyses (GH-A) (Hulett et al., 2000). Protein sequence alignment approaches in combination with secondary structure predictions indicated that heparanase contains sequences that are homologous to families 10, 39 and 51 of the GH-A, especially in terms of the active-site regions (Hulett et al., 2000). This clan of enzymes uses a general acid catalysis mechanism for the hydrolysis of glycosidic bonds. The mechanism requires two critical residues, a proton donor and a nucleophile,
both of which appear to be conserved in heparanase at Glu$^{225}$ and Glu$^{343}$, respectively (Hulett et al., 2000). Site-directed mutagenesis of these residues completely abolished heparanase activity, indicating that heparanase uses a catalytic mechanism characteristic of GH-A glycosyl hydrolases (Hulett et al., 2000).

### 3.2. Heparanase processing

Taking into account the multitude of polypeptides associated with HS on the cell surface and ECM and their ability to profoundly affect cell and tissue function, heparanase activity and bioavailability should be kept tightly regulated. Regulation at the transcriptional level represents one type of control mechanism. Regulation at the post-translational level, namely heparanase processing, cellular localization and secretion are now recognized as additional key regulatory mechanisms. A major 50 kDa protein is detected in cell lysates following transfection and over-expression of the heparanase cDNA, correlating with high levels of enzymatic activity (Vlodavsky et al., 1999). In contrast, a 65 kDa protein was found in the cell conditioned medium, raising the possibility that the protein is first synthesized as a latent proenzyme, which is then activated by proteolytic processing. Purifying human heparanase to homogeneity allowed Fairbanks et al. (1999) to determine the N-terminus sequence of the 50 kDa protein. Interestingly, the purified heparanase preparation was noted to include an 8 kDa protein and further analysis revealed that this protein is derived from the N-terminus region (Gln$^{56}$–Glu$^{109}$) of heparanase, immediately next to the signal sequence (Fairbanks et al., 1999). This finding led the authors to suggest that active heparanase is a heterodimer composed of the 8 and 50 kDa subunits. Indeed, attempts to express the truncated 50 kDa (Lys$^{158}$–Ile$^{543}$) protein alone yielded no enzymatic activity (Hulett et al., 2000), indicating that an N-terminus sequence is required. This hypothesis was confirmed by co-transfection and immunoprecipitation approaches, convincingly demonstrating that the two subunits are associated with each other, and that enzymatic activity is only obtained by co-expression of both the 8 and 50 kDa subunits (Levy-Adam et al., 2003; McKenzie et al., 2003). Multiple sequence alignment and secondary structure prediction suggest that heparanase adopts a TIM barrel fold, similar to other glycosyl hydrolases (Hulett et al., 2000). This fold motif usually consists of eight alternating $\alpha$-helices and $\beta$-strands. Within the heparanase 50 kDa subunit, clear homology was noted for only six $\alpha/\beta$ units, leading Nardella et al. to suggest that the two other units are contributed by the 8 kDa subunit. Indeed, structural prediction revealed the presence of a $\beta/\alpha/\beta$ element in the 8 kDa subunit, which may thus contribute the missing TIM barrel units (Nardella et al., 2004). These and other (McKenzie et al., 2003) studies, indicated that the linker domain (Ser$^{110}$–Gln$^{157}$) inhibits heparanase activity and needs to be fully removed. Adopting site-directed mutagenesis approach to identify amino acids essential for cleavage at Glu$^{109}$–Ser$^{110}$ (site 1) and Gln$^{157}$–Lys$^{158}$ (site 2), Abboud-Jarrous et al. (2005) reported that none of the mutations generated at site 1 and its flanking regions had an effect on heparanase processing and activity. In contrast, substitution of Tyr$^{156}$ (site 2) by alanine or glutamine rendered heparanase inactive and improperly processed (Abboud-Jarrous et al., 2005). Subsequent studies revealed that a bulky hydrophobic amino acid (i.e., tyrosine) at position 2 (P2) of the cleavage site (Gln$^{157}$–Lys$^{158}$) is absolutely required for heparanase processing and activation, resembling the cleavage specificity of cathepsin L (Abboud-Jarrous et al., 2005). Indeed, incubation of purified latent 65 kDa heparanase with cathepsin L yielded properly processed and active heparanase, composed of the 50 and 8 kDa subunits. Processing and activation of the pro-enzyme by intact cells and in a cell free system was inhibited in the presence of a specific, cell permeable inhibitor of cathepsin L (Abboud-Jarrous et al., 2005). It is likely that cathepsins other than cathepsin L may activate pro-heparanase, possibly in a cell- and tissue-dependent manner. Of note, impaired hair follicle morphogenesis and cycling, and abnormal bone development were noted in cathepsin L-deficient and cathepsin L knockout mice (Benavides et al., 2002; Potts et al., 2004). Intriguingly, heparanase over-expression in the skin and bone of transgenic mice resulted in the exact opposite phenomena, namely enhanced hair follicle cycling and hair growth (Zharia, Philp et al., 2005; Zharia, Žilka et al., 2005), and increased trabecular connectivity and bone mass (Kram et al., 2006), suggesting that alterations associated with cathepsin L deficiency are due, in part, to the possible lack of heparanase activity. Studies are underway to elucidate the effect of cathepsin L deficiency on heparanase function.

### 3.3. Lysosomal processing

An important issue is the nature of the processing organelle. Exogenously added pro-heparanase rapidly interacts with cells in culture, followed by internalization and conversion into a highly active enzyme, raising the possibility that processing occurs at the cell surface (Nadav et al., 2002; Vlodavsky & Friedmann, 2001). Recent findings indicated, however, that heparanase pro-
cessing occurs intracellularly (Gingis-Velitski, Zetser, Kaplan et al., 2004; Vreys et al., 2005; Zetser et al., 2004), pointing to acidic vesicles, most likely lysosomes, as the processing organelle. Following exogenous addition, heparanase was noted to reside within perinuclear endocytic vesicles identified as late endosomes (Nadav et al., 2002) and lysosomes (Goldshmidt et al., 2002). Applying anti-heparanase antibodies that distinguish between the latent and processed heparanase forms, we have demonstrated that not only the processed, but also the 65 kDa latent heparanase was localized in endocytic vesicles, indicating that processing does not take place at the cell surface (Zetser et al., 2004). Complete inhibition of heparanase processing by chloroquine and bafilomycin A1, reagents that raise the pH of acidic vesicles and thus inhibit the enzymatic activity of resident enzymes, further points to acidic vesicles as the heparanase processing site (Zetser et al., 2004). Taking this notion a step further, Cohen, Atzmon, Vlodavsky, and Ilan (2005) utilized a cell fractionation approach and demonstrated that lysosomal/endosomal, but not cytoplasmic preparation is capable of heparanase processing, yielding an active enzyme. Moreover, processing by the lysosomal/endosomal preparation was most efficient at acidic pH conditions (pH 4–5) (Cohen et al., 2005), typical of the lysosomal compartment. These results and the ability of cathepsin L, a characteristic lysosomal enzyme, to properly process and activate pro-heparanase, strongly support the lysosomal compartment as the site of processing. It should be noted, however, that most studies were performed with transfected cells engineered to over express heparanase, or in response to exogenous addition of the latent enzyme, situations that may not fully reflect the physiological conditions.

3.4. Secretion, trafficking and extracellular retention

In spite of its localization to a highly active protein degradation environment such as the lysosome, heparanase exhibits a half life of about 30 h (Gingis-Velitski, Zetser, Kaplan et al., 2004), relatively long compared with a $t_{1/2}$ of 2–6 h, and 25 min of transmembrane and GPI-anchored HSPGs, respectively (Egeberg, Kjeken, Kolset, Berg, & Prydz, 2001). Residence and accumulation of heparanase in late endosomes and lysosomes may indicate that the enzyme functions in physiological turnover of cellular HSPGs (Fuller, Chau, Nowak, Hopwood, & Meikle, 2005). Being not readily accessible to its extracellular substrate, suggests the existence of regulatory mechanism(s) by which intracellular, lysosomal heparanase is secreted in response to local or systemic cues. Recent observations may support the occurrence of such a scenario. For example, treatment of human microvascular endothelial cells (EC) with the pro-inflammatory cytokines TNFα and IL-1β resulted in a marked increase of heparanase secretion (Chen et al., 2004). Secretion of heparanase in response to TNFα was also noted in human peripheral T-cells (Sotnikov et al., 2004). Interestingly, TNFα and IL-1β had no effect on heparanase secretion from tumor-derived cells, suggesting that effective stimuli may vary among cell types and biological settings. Instead, nucleotides, such as ATP, ADP and adenosine were most effective in stimulating secretion of active heparanase by tumor cells (Shafat, Vlodavsky, & Ilan, 2006). Regarded as a universal source of metabolic energy, extracellular ATP, as well as other nucleotides are capable of initiating signaling cascades through two classes of P2 receptors: P2X, which has an intrinsic activity of ion channel; and P2Y, a G-protein coupled receptor (Communi, Janssens, Suarez-Huerta, Robaye, & Boeynaems, 2000). P2Y receptor activation is coupled to phospholipase C and adenylylate cyclase, leading to PKC and PKA activation (Abbracchio & Burnstock, 1998; Communi et al., 2000; van der Weyden, Conigrave, & Morris, 2000). Remarkably, each and every cell line examined responded to nucleotides (ATP, ADP, adenosine) by a stimulated secretion of active heparanase (Shafat et al., 2006). Importantly, ATP exerted its maximal effect at a physiological concentration (1 μM) (Gordon, 1986), emphasizing the biological relevance of this mediator, and heparanase secretion was inhibited by PKC inhibitors and P2Y receptor antagonists. Several lines of evidence suggest that the secreted heparanase originated from intracellular pools, most likely endosomes and lysosomes. The kinetics of heparanase secretion elicited by ATP resembled that of the lysosomal enzyme cathepsin D, supporting the notion that both enzymes were secreted from intracellular vesicles. Moreover, immunofluorescence staining revealed a clear transition in the localization of heparanase-positive vesicles toward the cell periphery, in response to stimulation with ATP (Shafat et al., 2006). Thus, although not considered as typical secretory vesicles, lysosomes may secrete their content under certain conditions and in response to the proper stimuli, in agreement with elevated levels of secreted cathepsins found in several human malignancies (Turk, Kos, & Turk, 2004; Turk, Turk, & Turk, 2000). The need for a proper stimulus is emphasized by studies showing that overexpression of heparanase in cancer cells is not necessarily associated with enhanced secretion, and that the enzyme is rarely secreted by normal cells. An exception are platelets and mast cells in which the enzyme accumulates in secretory granules...
and is released in response to activation and degranulation. It has been suggested, but not proven, that the platelet enzyme contributes to tumor metastasis as a result of platelets aggregating with circulating malignant cells and by virtue of their ability to identify small gaps between adjacent endothelial cells (Vlodavsky et al., 1992).

3.5. Cellular uptake

Apart of storage in the endosomal/lysosomal compartment, efficient uptake of exogenous heparanase by primary fibroblasts and EC, as well as by tumor-derived cells (Gingis-Velitski, Zetser, Flugelman, Vlodavsky, & Ilan, 2004; Gingis-Velitski, Zetser, Kaplan et al., 2004; Nadav et al., 2002; Zetser et al., 2004) provides an additional mechanism that limits retention of the enzyme extracellularly. Several lines of evidence indicate that heparanase uptake is mediated by cell surface HS. We have demonstrated that addition of heparin or xylosides results in accumulation of heparanase in the culture medium of heparanase transfected cells. Heparanase uptake was attenuated in HS-deficient cells and in cells that were treated with bacterial heparinase, but not with chondroitinase ABC. In addition, transfection and overexpression of heparanase in HS-deficient cells resulted in accumulation of the latent pro-enzyme in the culture medium, concomitant with decreased levels of the intracellular processed enzyme (Gingis-Velitski, Zetser, Kaplan et al., 2004). This result suggests that intracellular accumulation of processed heparanase occurs following uptake of the secreted latent protein (Gingis-Velitski, Zetser, Kaplan et al., 2004) (Fig. 2). Sequence alignment of heparin binding domains in the heparanase molecule revealed the existence of two domains that match consensus sequences for heparin binding. These were mapped at Lys158–Asp162 at the N-terminus of the 50 kDa heparanase subunit, and at Pro271–Met278 (Levy-Adam et al., 2005). A peptide containing the Lys158–Asp162 sequence (KKDC) exhibited firm binding to heparin and HS, and inhibited both heparanase uptake and enzymatic activity, most likely due to competition with the HS substrate (Levy-Adam et al., 2005). Furthermore, heparanase deletion mutants lacking each of the heparin binding domains exhibited no enzymatic activity. Deletion of the KKDC sequence (65Δ15) resulted in intracellular accumulation of the 65 kDa pro-enzyme that failed to get secreted. Deletion of the Pro271–Met278 sequence (65Δ10) led to accumulation of the pro-enzyme in the cell conditioned medium (Levy-Adam et al., 2005), further supporting a critical role for HS in heparanase uptake and processing. Notably, the KKDC sequence, and specifically lysine158 and lysine159 involved in interaction of the enzyme with heparin/HS (Levy-Adam et al., 2005), appeared to reside in close proximity to Glu225 and Glu343, comprising the enzyme’s active site in a micro pocket domain (Hulet et al., 2000) (Fig. 1). The predicted 3D model further emphasizes the micro pocket region and, in particular, the KKDC sequence (Fig. 1, HBD1) as a valid target for the development of heparanase inhibiting molecules. More recently, Vreys et al. (2005) have identified two additional cell surface receptors that mediate heparanase uptake, namely the low-density lipoprotein receptor-related protein (LRP) and the mannanse 6-phosphate receptor. Binding affinities to each component have not been determined and the precise contribution of each receptor species to heparanase uptake is yet to be demonstrated. Collectively, the above described studies clearly emphasize the complexity and tight regulation of heparanase expression, processing and secretion (Fig. 2), supporting its...
Fig. 2. A schematic presentation of a proposed model for heparanase biosynthesis, processing and trafficking. Pre-pro-heparanase is first targeted to the ER lumen via its own signal peptide (Met1–Ala35, 1). The 65 kDa pro-heparanase is then shuttled to the Golgi apparatus, and is subsequently secreted via vesicles that bud from the Golgi (2), a step that is specifically inhibited by Brefeldin A (BFA) (Nadav et al., 2002). Once secreted, heparanase rapidly interacts with cell membrane HSPGs such as syndecan-family members (Gingis-Velitski, et al., 2004), mannose-6 phosphate receptor or LRP (Vreys et al., 2005), followed by a rapid endocytosis of the heparanase-HSPG complex (4) that appears to accumulate in endosomes (Nadav et al., 2002). This step is inhibited by cytochalasin D (Cyto. D (Nadav et al., 2002), heparanase (Gingis-Velitski et al., 2004b) or heparin (Gingis-Velitski, et al., 2004). Conversion of endosomes to lysosomes results in heparanase processing and activation (5) that, in turn, participates in the turnover of HS side chains in the lysosome. Heparanase processing and activation is specifically inhibited by chloroquine and Bafilomycin A, inhibitors of lysosomal proteinases. Typically, heparanase appears at perinuclear lysosomal vesicles (6). Such a trafficking route may be bypassed by several potential ways, such as direct conversion of secretory vesicles to endosomes (dashed arrow). Lysosomal heparanase may translocate to the nucleus, where it affect gene transcription, thus contributing to a more differentiated state of carcinoma cells (Ohkawa et al., 2004; Takaoka et al., 2003) (7), or can get secreted in response to local or systemic cues. Secretion of active heparanase heterodimer is inhibited by PKC inhibitors (Bis), and P2Y receptor antagonists (MRS, PPADS) (Shafat et al., 2006) (8). The latent secreted heparanase can also interact with heparanase-binding protein (HBP) and activate signaling components such as Akt, p38, Pyk2 and integrins, leading to enhanced cell adhesion, migration, VEGF induction and angiogenesis (Gingis-Velitski, et al., 2004; Goldshmidt et al., 2003; Sotnikov et al., 2004; Zetser et al., 2003, 2006) (9).

potency and significance in normal and pathological conditions.

4. Heparanase and cancer progression

4.1. Pro-angiogenic properties

HSPGs are prominent components of blood vessels, and HSPG degrading enzymes have long been implicated in a number of angiogenesis-related cellular processes. A critical early event in the angiogenic process is degradation of the subendothelial basement membrane (BM), followed by endothelial cell (EC) migration toward the angiogenic stimulus. Similar to its involvement in tumor cell dissemination, it is conceivable that by degrading HS in the BM, heparanase may directly facilitate EC invasion and sprouting. Indeed, heparanase expression by bFGF-stimulated, bone marrow-derived EC was demonstrated by RT-PCR (Elkin et al., 2001). Immunohistochemistry of tumor specimens revealed heparanase staining of EC in capillaries, but not mature blood vessels (Elkin et al., 2001; Friedmann et al., 2000). Moreover, by releasing HS-bound angiogenic growth factors (i.e., bFGF, VEGF) from the ECM...
(Folkman et al., 1988), heparanase may indirectly facilitate EC migration and proliferation (Elkin et al., 2001; Vlodavsky et al., 1996). In fact, given the multitude of biological mediators that are sequestered by HS in the ECM (Vlodavsky, Bar-Shavit, Ishai-Michaeli, Bashkin, & Fuks, 1991), heparanase activity liberates a number of active molecules that may act cooperatively or synergistically to promote neovascularization. Moreover, HS fragments released by heparanase from the cell surface stimulate the mitogenic activity of bFGF (Elkin et al., 2001) and possibly other pro-angiogenic factors. Heparanase also releases growth factor-HS saccharide complexes from cell surfaces, although it has not been demonstrated whether such ‘liberated’ complexes are more active than counterparts that remain attached to membrane HSPGs.

Wound healing orchestrates multiple cell types (i.e., neutrophils, macrophages, fibroblasts, keratinocytes, endothelial cells), soluble (i.e., growth factors, cytokines, chemokines) and insoluble (ECM components) mediators in a complex sequence of events. Orchestration and regulation of the rapidly developing new tissue observed in wound healing depend not only on cells and bioactive polypeptides, but also on the ECM microenvironment, and require new blood vessel formation to nourish the newly formed granulation tissue. Elevated heparanase expression was observed in the wound granulation tissue and blood vessels (Zcharia, Zilka et al., 2005). Heparanase contribution to wound healing and wound angiogenesis has been demonstrated in several experimental settings. Increased amounts of heparanase were found in the wound fluid of heparanase transgenic (hpa-tg) versus control mice (Zcharia, Zilka et al., 2005), in agreement with heparanase expression in healing wounds. Moreover, elevated heparanase levels in the wound fluid correlated with a comparable elevation of bFGF (Zcharia, Zilka et al., 2005), providing an in vivo support for the ability of heparanase to release heparin-binding pro-angiogenic factors. Enhanced and persistent wound angiogenesis was further demonstrated in the hpa-tg mice by applying magnetic resonance imaging (MRI) (Zcharia, Zilka et al., 2005). This effect was inhibited by non-anti-coagulant glycol-split heparin (ST1514), shown to inhibit heparanase enzymatic activity at nM concentrations (Naggi et al., 2005) (see below), thus clearly supporting a role for heparanase in wound angiogenesis (Zcharia, Zilka et al., 2005). In a rat/flap punch model, topical application of highly active recombinant heparanase improved wound healing by 40% (Zcharia, Zilka et al., 2005) and enhanced wound angiogenesis (Elkin et al., 2001). Measurements taken in the area of flap incisions revealed a significant increase in epithelium thickness (Zcharia, Zilka et al., 2005), suggesting that heparanase promotes keratinocyte proliferation due to an improved bioavailability of factors such as KGF and HB-EGF. In addition, immunostaining of wound sections with anti-smooth muscle actin (SMA) antibody revealed a seven-fold increase in SMA-positive blood vessels in response to heparanase treatment (Zcharia, Zilka et al., 2005). Thus, heparanase accelerates wound healing by enhanced migration and proliferation of keratinocytes and stimulation of wound blood vessel formation and maturation. The coordinate, simultaneous release of a combination of HS-bound growth factors (i.e., bFGF, VEGF, HB-EGF, KGF) is unique to heparanase and may account for its efficient neovascularization and wound healing promoting effect. It should be noted that wound healing is only one example for the involvement of heparanase in tissue remodeling and neovascularization. Thus, heparanase transgenic mice (hpa-tg) exhibit excess branching and widening of mammary gland ducts, and accelerated rate of hair growth, both accompanied by enhanced vascularization (Zcharia et al., 2004; Zcharia, Philp et al., 2005).

4.2. Non-enzymatic functions associated with angiogenesis, cell survival and migration

Applying heparanase that lacks enzymatic activity due to point mutations (Glu225, Glu343) in its active site, we have noted that heparanase exerts also non-enzymatic activities, independent of its involvement in ECM degradation and alterations in the extracellular microenvironment. For example, cell surface expression of enzymatically inactive heparanase elicits a firm cell adhesion, reflecting an involvement in cell–ECM interaction (Goldshmidt et al., 2003). Moreover, as described below, inactive heparanase enhances Akt signaling and stimulates PI3K- and p38-dependent endothelial cell migration and invasion (Gingis-Velitski, Zetser, Flugelman et al., 2004). It also promotes VEGF expression via the Src pathway (Zetser et al., 2006). At present, no information is available on protein domains responsible for the non-enzymatic functions of the heparanase molecule, nor on the putative heparanase receptor that appears to mediate these effects.

4.2.1. Akt activation

Heparanase over-expression in human U87 glioma (Zetser, Bashenko, Miao, Vlodavsky, & Ilan, 2003), HT 29 colon carcinoma (Doviner et al.), CAG myloma (Yang et al., 2005), MCF7 (Cohen et al., 2006), MDA-MB-231 (Kelly et al., 2005) and MDA-MB-435 (Zetser et al., 2006) breast carcinoma cells correlated with enhanced
xenograft tumor growth and vascularization. Using the RIP-Tag2 tumor model, Joyce, Freeman, Meyer-Morse, Parish, and Hanahan, 2005 have recently demonstrated elevated levels of heparanase mRNA and protein upon the transition from normal to angiogenic islets, which further increased when solid tumors were detected. These studies support the notion that heparanase not only facilitates tumor metastasis, but also contributes to the angiogenic switch and subsequent growth of the primary tumor. Enhanced tumor progression correlated with elevation in blood vessel density, revealed by staining with anti-PECAM-1 antibodies, as well as by MRI analysis (Cohen et al., 2006). At the molecular level, heparanase over-expression was noted to facilitate cell adhesion and migration of tumor cells, primary EC and T lymphocytes, mediated, at least in part, by β1-integrin and Rac activation (Goldshmidt et al., 2003; Sotnikov et al., 2004; Zetser et al., 2003). Heparanase over-expression in U87 glioma, as well as in several other tumor derived cells, correlated with enhanced Akt/PKB phosphorylation levels (Zetser et al., 2003). Moreover, exogenous addition of heparanase to primary EC markedly stimulated Akt phosphorylation (Gingis-Velitski, Zetser, Flugelman et al., 2004). This effect occurred both in the presence or absence of HS on the cell surface, was independent of heparanase enzymatic activity, and was augmented by heparin (Gingis-Velitski, Zetser, Flugelman et al., 2004). At the cellular level, heparanase addition stimulated PI 3-kinase-dependent EC migration and invasion, and significantly improved EC rearrangement into lumen containing tube-like structures (Gingis-Velitski, Zetser, Flugelman et al., 2004). These observations implicate that heparanase is capable of eliciting angiogenic responses by a direct effect on EC. The ability of heparanase to stimulate Akt suggests that heparanase may protect tumor cells from apoptosis (Cohen et al., 2006), although the survival promoting mechanism has not been sufficiently elucidated. The ability of exogenously added heparanase to activate signal transduction cascades, and its augmentation by heparin may indicate the existence of a cell surface heparanase receptor (Fig. 2, HBP), possibly LRP (Vreys et al., 2005), yet this aspect awaits further research and proper confirmation.

4.2.2. VEGF induction and Src activation

In addition to the above described pro-angiogenic effects attributed to heparanase enzymatic and non-enzymatic activities, heparanase is also closely involved in VEGF gene regulation. Transfection and over-expression of heparanase in rat C6 glioma, MDA-MB-435 human breast carcinoma and human embryonic kidney HEK293 cells, were accompanied by a three- to six-fold increase in VEGF mRNA and protein levels, correlated with enhanced Matrigel and tumor xenograft vascularization (Zetser et al., 2006). Moreover, transfection of the highly metastatic mouse B16-BL6 melanoma cells with heparanase-specific siRNA resulted in a 75% decrease in heparanase mRNA and VEGF levels. This implies that endogenous heparanase is intimately involved in VEGF gene regulation. Interestingly, VEGF elevation by heparanase correlated with increased p38 phosphorylation levels, a signaling pathway implicated in VEGF induction (Zetser et al., 2006). Nonetheless, p38 inhibitors had no effect on heparanase-mediated VEGF up regulation, suggesting the operation of another signaling pathway(s) elicited by heparanase. Screening of several additional inhibitors led to the identification of Src, a kinase that was shown to modulate VEGF transcription (Ellis et al., 1998; Jiang, Agani, Passaniti, & Semenza, 1997; Mukhopadhyay et al., 1995), as a mediator of VEGF up regulation by heparanase. Moreover, Src inhibitors prevented VEGF induction by heparanase and significantly attenuated cell migration enhanced by heparanase, positioning Src as a critical down stream component that mediates heparanase functions (Zetser et al., 2006). VEGF induction and Src activation require heparanase secretion that was recapitulated by exogenous addition of the enzyme. Importantly, Src activation was noted also upon exogenous addition of point mutated (Glu225, Glu343) heparanase that lacks enzymatic activity. Thus, heparanase exerts enzymatic activity-dependent (i.e., release of bFGF) and independent (i.e., VEGF induction) pro-angiogenic effects. It therefore appears that in addition to its pro-metastatic function (see below), heparanase affects several key components in tumor progression, resulting in increased blood vessel density and maturation, enhanced tumor cell motility and activation of signaling mediators that govern tumor cell proliferation (i.e., Src) and survival (Akt). Collectively, these effects position heparanase as an attractive target for the development of anti-cancer drugs.

4.3. Pro-metastatic properties

Heparanase activity in tumor cells was initially investigated in B16 melanoma (Nakajima et al., 1983; Nakajima, Trimm, Di Ferrante, & Nicolson, 1984) and T-lymphoma cells (Vlodavsky, Fuks, Bar-Ner, Ariav, & Schirrmacher, 1983), resulting in HS fragments five to six times smaller than intact HS side chains. Heparanase activity was further characterized as an endoglucuronidase and found to be inhibited by species of heparin (Bar-Ner et al., 1985; Nakajima et al., 1984).
Heparanase activity correlated with the metastatic potential of mouse B16 melanoma and Eb lymphoma cells. Thus, sub lines with higher potential for metastasis and organ colonization exhibited a higher enzymatic activity than low- or non-metastatic cells. These early observations gained substantial support when specific molecular probes became available shortly after cloning the heparanase gene. Hulett et al. (1999) employed Northern blot analysis to study heparanase expression in cells and tissues, while Vlodavsky et al. (1999) utilized a transfection approach. In these studies, highly metastatic rat mammary adenocarcinoma cell lines (13762 MAT, DMBA-8A) were noted to express high levels of heparanase mRNA transcripts compared with their non-metastatic counterpart cells (Hulett et al., 1999). Subcutaneous inoculation of non-metastatic Eb lymphoma cells engineered to over express heparanase (hpa-Eb) resulted in a significant decrease in survival time of the mice due to a massive liver infiltration (Vlodavsky et al., 1999), further supporting the correlation between heparanase expression and the metastatic capacity of cancer cells. In a myeloma cell model, Yang et al. demonstrated that enhanced heparanase expression markedly induced spontaneous metastasis into various organs, depending on the site of primary tumor inoculation. Subcutaneously injected myeloma cells metastasized to the spleen, liver, lung and bone, while cells injected into the bone selectively disseminated to other bones (Yang et al., 2005). Moreover, vigorous bone resorption was noted in SCID mice following inoculation of heparanase-transfected MDA-231 human breast carcinoma cells into the mammary fat pad, although bone metastases were not detected (Kelly et al., 2005). It appears that heparanase over-expression in tumor cells can exert a systemic effect, resulting in elevation of soluble factors that stimulate osteolysis and, perhaps, progression of bone-homing tumors (Kelly et al., 2005; Sanderson et al., 2005). In other studies, siRNA and ribozyme technologies were employed to reduce heparanase expression levels in a specific manner. Transfection and stable expression of anti-heparanase ribozyme construct in human MDA-MB-435 breast carcinoma cells, known to express high levels of heparanase activity, or in Eb mouse lymphoma cells engineered to over express the human heparanase gene (hpa-Eb), resulted in a marked decrease in heparanase levels evaluated by RTPCR and heparanase enzymatic activity. This decrease correlated with 55–65% reduction in cellular invasion through a reconstituted basement membrane (Matrigel) (Edovitsky, Elkin, Zcharia, Peretz, & Vlodavsky, 2004). Moreover, mice inoculated (s.c) with hpa-Eb lymphoma cells transfected with anti-heparanase ribozyme exhibited a marked decrease in liver metastasis and survived significantly longer than mice inoculated with cells transfected with control ribozyme. Similarly, lung colonization of B16-BL6 melanoma cells was markedly (>90%) reduced applying cells transfected with anti-heparanase siRNA due to a marked inhibition of both heparanase gene expression and enzymatic activity, naturally expressed by these cells (Edovitsky et al., 2004). Subcutaneous primary tumors produced by hpa-Eb cells expressing the anti-heparanase ribozyme were less vascularized, supporting the pro-angiogenic function of the enzyme. Altogether, both over-expression and silencing of the heparanase gene clearly indicate that heparanase not only enhances cell dissemination, but also promotes the establishment of a vascular network that accelerates primary tumor growth and provides a gateway for invading metastatic cells. High levels of heparanase expression mostly correlate with increased primary tumor growth and metastatic potential (Cohen et al., 2006; Kelly et al., 2005; Vlodavsky et al., 1999).

Applying U87 human glioma cells, we have noted, however, that exceedingly elevated heparanase expression levels correlated with decreased proliferation rate, increased cell spreading and formation of a tight monolayer (Zetser et al., 2003). Moreover, in a xenograft tumor model, moderate heparanase expression levels significantly enhanced tumor growth, while high expression levels inhibited tumor progression. Taking into account the important role of HS in growth factor signaling, this apparently unexpected observation may be due to insufficient levels of HS remaining on the cell surface. Thus, depending on its expression levels, heparanase may promote or suppress cell proliferation and tumor progression.

While the studies described above provide a proof-of-concept for the pro-metastatic and pro-angiogenic capacity of heparanase, the clinical significance of the enzyme in tumor progression emerges from a systematic evaluation of heparanase expression in primary human tumors. Immunohistochemistry, in situ hybridization, RT-PCR and real time-PCR analyses revealed that heparanase is up regulated in essentially all human tumors examined. These include carcinomas of the colon (Friedmann et al., 2000; Sato et al., 2004), thyroid (Xu et al., 2003), liver (El-Assal, Yamanoi, Ono, Kohno, & Nagasue, 2001), pancreas (Kim et al., 2002; Koliopanos et al., 2001; Rohloff et al., 2002), bladder (Gohji, Hirano et al., 2001; Gohji, Okamoto et al., 2001), cervix...
(Shinyo, Kodama, Hongo, Yoshinouchi, & Hiramatsu, 2003), breast (Maxhimer et al., 2002), gastric (Takaoka et al., 2003; Tang et al., 2002), prostate (Ogishima, Shiina, Breault, Tabatabai et al., 2005), head and neck (Beckhove et al., 2005; Mikami et al., 2001; Simizu, Ishida, Wierzbà, Sato, & Osada, 2003) and oral cavities, as well as multiple myeloma (Kelly et al., 2003), leukemia and lymphoma (Bitan et al., 2002). In most cases, elevated levels of heparanase were detected in about 50% of the tumor specimens, with a higher incidence in pancreatic (78%) and gastric (80%) carcinomas, and in multiple myeloma (86%). In all cases, the normal looking tissue adjacent to the malignant lesion expressed little or no detectable levels of heparanase, suggesting that epithelial cells do not normally express the enzyme. This is in agreement with the notion that under normal conditions heparanase expression is restricted primarily to the placenta, keratinocytes, lymphocytes, neutrophils, macrophages and platelets (Parish et al., 2001; Vlodavsky & Friedmann, 2001). In several carcinomas, most intense heparanase staining was localized to the invasive front of the tumor (Beckhove et al., 2005; Gohji, Okamoto et al., 2001; Ohkawa et al., 2004; Tang et al., 2002), supporting a role for heparanase in cell invasion. Furthermore, patients that were diagnosed as heparanase-positive exhibited a significantly higher rate of local and distant metastasis as well as reduced post-operative survival, compared with patients that were diagnosed as heparanase-negative (Gohji, Okamoto et al., 2001; Kelly et al., 2003; Rohloff et al., 2002; Sato et al., 2004; Takaoka et al., 2003; Tang et al., 2002). Collectively, these studies provide a strong clinical support for the pro-metastatic function of heparanase. Interestingly, patient’s survival was noted to correlates not only with heparanase levels, but also with its localization. In addition to localization in the cytoplasm, heparanase was also noted to assume nuclear localization, demonstrated by cell fractionation (Schubert et al., 2004), and by immunostaining of cultured cells (Schubert et al., 2004) and tumor biopsies (Ohkawa et al., 2004; Takaoka et al., 2003). Interestingly, nuclear localization was correlated with maintained cellular differentiation (Ohkawa et al., 2004) and favorable outcome of patients with gastric (Ohkawa et al., 2004; Takaoka et al., 2003) and head and neck (Dueck et al., submitted for publication) carcinomas, suggesting that heparanase is intimately involved in gene regulation. Whether gene transcription and maintained cellular differentiation is due to direct interaction of heparanase with the DNA, or is a consequence of heparanase-mediated nuclear-HS degradation is yet to be demonstrated. In addition, heparanase up regulation in primary human tumors correlated in some cases with tumors larger in size (El-Assal et al., 2001; Maxhimer et al., 2002; Tang et al., 2002), and with enhanced micro vessel density (El-Assal et al., 2001; Gohji, Hirano et al., 2001; Kelly et al., 2003; Sato et al., 2004; Shinyo et al., 2003; Watanabe, Aoki, Kase, & Tanaka, 2003), providing a clinical support for the pro-angiogenic function of the enzyme.

4.4. Heparin and cancer

Low-molecular-weight heparin (LMWH) appears to prolong survival in patients with cancer. In each of six recently published randomized-controlled trials (RCTs), four different types of LMWH increased the survival in patients with advanced cancer (Kakkar et al., 2004; Klerk et al., 2005; Rickles, 2006). The favorable results in cancer patients [generally a subset of larger studies of treatment of patients with venous thromboembolism (VTE)] could not be demonstrated in non-cancer patients in the same studies. Although VTE complicating cancer has an adverse effect on prognosis across all tumor types, death in these patients usually occurs because of cancer, not VTE (Sorensen, Mellemkjaer, Olsen, & Baron, 2000). Indeed, rather than preventing fatal pulmonary emboli in cancer patients, it seems more likely that LMWH has either direct effects on tumor growth and metastasis and/or indirect effects mediated by inhibition of thrombin generation, the latter of which has been widely implicated in the growth and metastasis of tumors (Rickles, Patierno, & Fernandez, 2003).

The use of native heparin as an anti-metastatic agent is limited due to its potent anti-coagulant activity. Non-anti-coagulant heparins are of clinical potential because they could be administered at higher doses, thereby fully exploiting the anti-metastatic component of heparin, and because they could be applied to cancer patients with bleeding complications, where the use of heparin is precluded. The mechanism by which non-anti-coagulant heparin inhibits metastasis is not fully understood. One possibility is that heparin inhibits metastasis by blocking platelet–tumor cell interactions, thereby inhibiting aggregates of tumor cells from lodging in the microvasculature (Borsig et al., 2001). Non-anti-coagulant heparin also inhibits selectin-mediated cell–cell interactions (Borsig, 2004) thus preventing tumor cell adhesion to the vascular endothelium and subsequent extravasation of blood borne cells. These effects, the inhibition of heparanase enzymatic activity and the encouraging animal studies and clinical trials, clearly warrant further investigation of non-anti-coagulant heparins as a promising therapeutic strategy for the inhibition of cancer metastasis (see below).
5. Inhibitory molecules and clinical considerations

Attempts to inhibit heparanase enzymatic activity were initiated already at the early days of heparanase research, in parallel with the emerging clinical relevance of this activity. More recently, with the availability of recombinant heparanase and the establishment of high-throughput screening methods, a variety of inhibitory molecules have been developed, including neutralizing antibodies, peptides, small molecules, modified non-anti-coagulant species of heparin, as well as several other polyanionic molecules, such as laminaran sulfate, suramin and PI-88 (Ferro, Hammond, & Fairweather, 2004; Simizu, Ishida, & Osada, 2004).

5.1. Chemically modified, non-anti-coagulant species of heparin

Animal studies using non-anti-coagulant species of heparin indicate that it is possible to separate the anti-metastatic and anti-coagulant activities of heparin. The use of native heparin as an anti-metastatic agent is limited due to its potent anti-coagulant activity. Non-anti-coagulant heparins are of clinical potential because they can be administered at higher doses, thereby fully exploiting the anti-metastatic component of heparin, and because they can be applied to cancer patients with bleeding complications, where the use of heparin is precluded. The mechanism by which non-anti-coagulant heparin inhibits metastasis is not fully understood. One possibility is that heparin inhibits metastasis by blocking platelet–tumor cell interactions, thereby inhibiting aggregates of tumor cells from lodging in the microvasculature (Borsig et al., 2001). Non-anti-coagulant heparin also inhibits selectin-mediated cell–cell interactions (Borsig, 2004) thus preventing blood borne cell adhesion to the vascular endothelium and subsequent extravasation. It stimulates tissue factor pathway inhibitor release and inhibits inflammatory responses (Thodiyil & Kakkar, 2002). These effects, the inhibition of heparanase enzymatic activity and the encouraging animal studies and clinical trials, clearly warrant further investigation of non-anti-coagulant heparins as a promising therapeutic strategy for the inhibition of cancer metastasis. The site of HS cleavage by heparanase is the β-glycosidic linkage of a glucoronic (GlcA) residue which must be flanked by N-sulfated or N-acetylated α-linked glucosamine (GlcN) residues. At least one O-SO$_3$ group is essential for efficient recognition by the enzyme (Ishida et al., 2004; Parish et al., 1999). HS/heparin and derived oligosaccharides must have a minimal octasaccharidic size to be good substrates for heparanase (Ishida et al., 2004; Parish et al., 1999). However, the enzyme can be efficiently inhibited also by shorter but more extensively sulfated oligosaccharides such as maltohexaose polysulfate (MHS) and phosphomannopentaose polysulfate (PI-88) (see below). As an analog of its natural HS substrate, heparin is commonly considered as a potent inhibitor of heparanase (Bar-Ner et al., 1987). This activity is attributed, in part, to its high affinity interaction with the enzyme and limited susceptibility to degradation, serving as an alternative substrate (Nasser et al., 2006). Early reports showed that heparin and some chemically modified species of heparin, inhibit experimental metastasis in animal models, while other related compounds (e.g., chondroitin sulfate, carrageenan-κ, hyaluronic acid) that lack heparanase inhibiting activity fail to exert an anti-metastatic effect (Nakajima, Irimura, & Nicolson, 1988; Parish, Coombe, Jakobsen, Bennett, & Underwood, 1987; Vlodavsky et al., 1994). Screening of heparin derivatives permitted to identify some of its structural features associated with inhibition of the enzyme. We have analyzed the heparanase-inhibiting effect of heparin derivatives differing in degrees of 2-O- and 6-O-sulfation, N-acetylation and glycol-splitting of non-sulfated uronic acid residues (Naggi et al., 2005). The contemporaneous presence of sulfate groups at O-2 of IdoA and O-6 of GlcN was not essential for effective inhibition of heparanase activity, provided that one of the two positions retains a high degree of sulfation. N-desulfation/N-acetylation led to a marked decrease in inhibitory activity, suggesting that at least one NSO$_3$ group per a disaccharide unit is involved in interaction with the enzyme. On the other hand, glycol-splitting of non-sulfated uronic acids dramatically increased the heparanase-inhibiting activity of N-acetylated heparin, irrespective of the degree of N-acetylation (Naggi, 2005; Naggi et al., 2005). Glycol-split derivatives of heparin are obtained by controlled periodate oxidation/borohydride reduction of natural or partially 2-O-desulfated heparins (Casu et al., 2002; Naggi, 2005; Naggi et al., 2005). Glycol-splitting of C2–C3 bonds of non-sulfated uronic acid residues was suggested to interfere with the biological interactions of heparin by providing flexible joints between its protein binding sequences (Naggi, 2005). It was found that N-acetylated heparins in their glycol-split forms inhibit heparanase as effectively as the corresponding N-sulfated derivatives. Glycol-split heparin efficiently inhibited heparanase driven DTH inflammation (Edovitsky et al., 2006) and wound angiogenesis (Zcharia, Zilka et al., 2005).

Whereas heparin and N-acetyl heparins containing unmodified GlcA residues inhibit heparanase by act-
ing, at least in part, as substrates, their glycol-split derivatives are no longer susceptible to cleavage by heparanase. It appears that formation of glycol-split residues generates three additional degrees of rotational freedom per each split residue, thus facilitating docking of heparin sequences to sites essential for heparanase enzymatic activity. Notably, glycol-split N-acetyl heparins exhibit a marked decrease in the ability to release bFGF from ECM and to stimulate its mitogenic activity. Moreover, glycol-splitting involves substantial loss of the anti-coagulant activity of heparin due to a complete loss of the heparin affinity for antithrombin (Casu et al., 2002). Collectively, the combination of high inhibition of heparanase, lack of anti-coagulant activity and low release/potentiation of ECM-bound bFGF, points to N-acetylated, glycol-split heparins as potential anti-angiogenic and anti-metastatic agents, more effective and specific than their counterparts with unmodified backbones (Naggi et al., 2005).

5.2. Sulfated phosphomannopentaose (PI-88)

Sulfated malto-oligosaccharides, ranging from di- to heptasaccharide, were prepared and tested for inhibitory activity against rat hepatoma heparanase (Ferro et al., 2004; Simizu, Ishida, & Osada, 2004). The two most potent inhibitors, which had activity comparable to heparin (IC50 = 1 µg/mL), were found to be sulfated maltohexaose (IC50 = 1.5 µg/mL) and the phosphosulfomannan PI-88 (IC50 = 2 µg/mL). PI-88 exhibits anti-angiogenic, anti-metastatic and anti-restenotic activities and is currently undergoing Phase II clinical trials for melanoma, myeloma and lung carcinoma. Continuous administration of PI-88 inhibits growth, vascularity and lymph node metastasis of mammary adenocarcinoma tumors in rats. This drug, which is composed primarily of sulfated phosphomannopentaose and phosphomannotetraose oligosaccharide units, is thought to exert its biological effects by blocking the enzymatic activity of heparanase and by interfering with the action of HS-binding growth factors such as fibroblast growth factor-1 (FGF-1), bFGF and vascular endothelial growth factor (VEGF) (Khachigian & Parish, 2004). PI-88 interferes with a relatively broad range of protein–HS interactions, making interpretation regarding specificity and mode of action, questionable, similar to other polyanionic compounds. PI-88 and other heparanase-inhibiting sulfated oligosaccharides and modified heparin derivatives may also be applied to suppress restenosis, autoimmunity and chronic inflammatory diseases.

To obtain homogeneous and structurally well defined HS mimetics, a family of totally synthetic molecules, namely sulfated linked cyclitols, was prepared (Ferro et al., 2004). Other series of compounds were phosphosulfomannans, sulfated spaced oligosaccharides, and sulfated oligomers of glycamino acids. The structure and heparanase-inhibiting effect of these and other compounds are described in detail in recent reviews by Ferro et al. (2004) and Simizu, Ishida, and Osada (2004).

5.3. Suramin

Suramin is a polysulfonated naphthyl urea, mimicking HS and having antineoplastic effects (Stein, 1993) attributed to its ability to inhibit growth and block angiogenesis. Suramin’s mechanism of growth inhibition was attributed to its ability to block growth factor binding to cell surface receptors, while its anti-angiogenic and anti-metastatic effects appear to be through blocking the activity of heparanase (Marchetti, Reiland, Erwin, & Roy, 2003; Nakajima et al., 1991). One reason that suramin has not been widely used is that it has significant toxic effects in humans, including neurotoxicity, renal toxicity, adrenal insufficiency and anti-coagulant-mediated blood dyscrasias (Figg et al., 1994; Stein, 1993). To avoid side effects, analogues of suramin have been generated and are undergoing evaluation. NF 227, NF 145 and NF 171 are three such analogues, all of which have been shown to inhibit heparanase-mediated angiogenesis in an animal model (Marchetti et al., 2003).

5.4. Non-carbohydrate heparin mimetic polymers

Synthetic, linear, non-carbohydrate polyanionic polymers have been studied for many years as heparin mimetics. A series of polymers of carboxylated phenols (Benezra, Ishai-Michaeli, Ben-Sasson, & Vlodavsky, 2002; Benezra et al., 2001) was tested for inhibition of heparanase-mediated degradation of HS in the subendothelial ECM. Several compounds, including a polymer of (4-hydroxyphenoxo) acetic acid of MW ~ 5800, designated RG-13577, showed almost complete inhibition of heparanase activity at a concentration of 2.5 µg/mL (Benezra et al., 2002). A series of poly(N-acryl amino acids) ranging in MW from 3000 to 60,000 with lipophilic or polar side chains were synthesized by radical polymerization of N-acryl amino acid monomers (Bentonila et al., 2000). Poly(N-acryl amino acids) containing hydroxyl groups were also sulfonated, yielding polymers that were comparable to heparin in their heparanase inhibitory potency.
5.5. Peptides competing with the heparin/HS binding domains of heparanase

We identified three potential heparin-binding domains in the heparanase molecule and provided evidence that one of these domains is mapped at the N-terminus of the 50 kDa heparanase subunit (Levy-Adam et al., 2005). A peptide corresponding to this region (Lys 158-Asn 171) physically associates with heparin and HS and inhibits heparanase enzymatic activity in a dose-responsive manner. Furthermore, antibodies directed to this region inhibited heparanase activity and a deletion construct lacking this domain exhibited no enzymatic activity (Levy-Adam et al., 2005). We have introduced a cysteine residue at the peptide C-terminus and found that oxygenation and spontaneous dimerization of the KKDC peptide significantly improved its heparanase-inhibiting activity.

Studies on processing and activation of the 65 kDa heparanase proenzyme revealed that the linker peptide must be totally removed in order to enable interaction between heparanase and its substrate (Nardella et al., 2004). It was demonstrated that the linker segment, or even a small (~1 kDa) peptide corresponding to its C-terminus inhibit heparanase enzymatic activity (McKenzie et al., 2003; Nardella et al., 2004), most likely by hindering accessibility of the HS substrate to the enzyme’ active site. While predicted model structures do provide important information (Fig. 1), it is limited by the relatively low sequence homology with other glycosyl hydrolases, and awaits further conformation by crystallization and X-ray analysis.

5.6. Small molecules

Small molecule heparanase inhibitors, exemplified by 1H-isoindole-5-carboxylic acid and benzoxazol-5-yl-acetic acid, were reported. A novel class of 1-[4-(1H-benzoimidazol-2-yl)-phenyl]-3-[4-(1H-benzoimidazol-2-yl)-phenyl]-ureas were recently described as potent inhibitors of heparanase (Ferro et al., 2004). Among these, compound 1,3-bis-[4-(1H-benzoimidazol-2-yl)-phenyl]-urea displayed a potent heparanase inhibitory activity (IC50 = 0.075 μM) in vitro and a good efficacy in a B16 experimental metastasis model (Pan et al., 2006).

5.7. Other inhibitory strategies

An attractive approach for the inhibition of heparanase is the development and use of neutralizing monoclonal antibodies to the protein. A monoclonal antibody has been reported which effectively abolishes the activity of recombinant heparanase when used at a protein:antibody ratio of 1:10 (Ferro et al., 2004). A neutralizing polyclonal antibody directed against a peptide corresponding to the heparanase active site (residues G215–D234) effectively inhibited restenosis in a rat model (Myler, Lipke, Rice, & West, 2006). Random, high-throughput screening of chemical libraries and microbial metabolites, and rational design of compounds that block the heparanase active site or ligand-binding domain are among the other approaches applied to develop effective heparanase inhibitors (Ferro et al., 2004; Pan et al., 2006; Simizu, Ishida, & Osada, 2004). Natural endogenous heparanase inhibitors may also be identified. It would seem plausible that further defining the heparanase substrate specificity, catalytic and non-catalytic activities, as well as the enzyme X-ray crystal structure would be invaluable for pursuing a more ‘rational’ approach to develop effective and highly specific heparanase inhibiting molecules.

6. Conclusions and perspectives

Although a significant progress has been made during the last several years in understanding heparanase biology, there is much to be learned. Accumulation of compelling evidence implies that the enzyme is up regulated in primary human tumors and inversely correlates with survival rate of cancer patients post-operation. While angiogenesis is the primary ‘suspect’ that governs heparanase-mediated tumor progression, this mode of action and the related clinical applications await further confirmation and require new molecular tools such as small inhibitory molecules, neutralizing antibodies, and more stable, flexible and specific heparanase-inhibiting species of heparin and other saccharides. Although a few mechanisms that promote heparanase induction under pathological conditions were reported, heparanase regulation at the transcriptional level requires a further investigation. The ability of heparanase to function in an apparently enzymatic independent manner, noted in several experimental settings (Gingis-Velitski, Zetser, Flugelman et al., 2004; Goldshmidt et al., 2003; Sotnikov et al., 2004; Zetser et al., 2003, 2006), is intriguing and affects the way the protein is envisioned. Thus, while attention was mainly focused on compounds that inhibit heparanase enzymatic activity, no information is available on protein domains responsible for the non-enzymatic functions of the heparanase enzyme. In this respect, identification of a putative heparanase receptor is a major future challenge. Another important objective is the establishment of a reliable diagnostic assay
to monitor heparanase levels and activity in plasma and urine of cancer patients by means of a sensitive, high-throughput activity assay and ELISA method. The secreted nature of the enzyme and its induction in primary human tumors predict that under certain conditions the protein is present in body fluids. It is conceivable that elevated levels of heparanase are found in the plasma and/or urine of cancer patients as well as in other pathological disorders such as diabetes (Katz et al., 2002; Levidiotis, Freeman, Tikellis et al., 2004; Shafat et al., 2006). Induction of heparanase already at early phases of cancer progression supports an important diagnostic and, possibly, prognostic value of such assays.

Recent publications clearly imply that heparanase may be involved in pathological conditions other than cancer. An intriguing example is the observation that heparanase over expressing transgenic mice escaped amyloid deposition in experimental models of inflammatory-associated amyloidosis (Li et al., 2004). The possible involvement of heparanase in degenerative diseases such as Alzheimer’s disease is only starting to emerge and should be perused, taking into account the increasing significance of these illnesses. Likewise, the causal involvement of heparanase in diabetic nephropathy (Levidiotis et al., 2005), autoimmunity (Lider et al., 2009) and inflammatory disorders (Edovitsky et al., 2006) should be investigated. Heparanase knock-out mice are being generated by several groups in order to further elucidate the involvement of heparanase in the above described normal and pathological processes. Such mice will provide most valuable information on the role of heparanase in normal development, tumor progression and inflammation. Heparanase knock-out mice will be utilized, for example, to identify possible side effects of heparanase gene silencing and other inhibitory strategies, and to investigate the contribution of host-derived heparanase to tumor development.

Functional domains other than the basic heterodimer structure (Fairbanks et al., 1999; Levy-Adam et al., 2003; McKenzie et al., 2003) and amino acids (Glu225, Glu343) critical for the enzyme catalytic activity (Hulett et al., 2000) have not been so far identified in the heparanase protein, making screens for inhibitory molecules random in nature. The identification of heparin binding domains (Levy-Adam et al., 2005) and the ability of the corresponding KKDC peptide, especially when applied as a dimer, to inhibit heparanase enzymatic activity (Levy-Adam et al., 2005; Zetser et al., 2004), clearly emphasize the need for crystallization and accurate understanding of the 3D structure of the enzyme toward an efficient drug development program.

While it is now well accepted that a single active heparanase enzyme is expressed by mammals, heparanase splice variants have recently been characterized in the Mole rat (Nasser et al., 2005), although their role has not been established. The heparanase system may be envisaged to include heparanase 1 and its splice variants, the three splice variants of heparanase 2 (McKenzie et al., 2000), the heparanase processing protease, and, possibly, the heparanase cell surface receptor. Gain of more information will enable the development of new inhibitory strategies directed against the enzymatic and non-enzymatic functions of heparanase, altogether offering better therapeutic opportunities.

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References


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