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Review

Heparanase involvement in physiology and disease

N. J. Nasser

Department of Oncology, Shaare Zedek Medical Center, P.O.B. 3235, Jerusalem 91031 (Israel),
Fax: +972 2 6521431, e-mail: nicola.nasser@gmail.com

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Abstract. Heparanase is an endoglycosidase that degrades heparan sulfate on the cell surface and extracellular matrix. The physiological functions of heparanase include heparan sulfate turnover, embryo development, hair growth, and wound healing. Heparanase is implicated in a variety of pathologies, such as tumor growth, angiogenesis, metastasis, inflammation, and glomerular diseases. Heparanase overexpression in a variety of malignant tumors suggests that it could be a target for anti-cancer therapy.

Keywords. Cancer progression, extracellular matrix, heparin, heparanase, angiogenesis, alternative splicing, metastasis, hypoxia.

Heparan sulfate proteoglycans (HSPGs)

Structure

Proteoglycans consist of a core protein to which one or more glycosaminoglycan chains are attached [1 – 5]. Glycosaminoglycans are unbranched polysaccharides, composed of repeating disaccharide units incorporating an aminosugar and uronic acid. Glycosaminoglycan chains (heparan sulfate (HS), chondroitin sulfate, dermatan sulfate or keratan sulfate) are linked to the core protein via a tetrasaccharide linker that has the sequence xylose-galactose-galactose-uronic acid. The tetrasaccharides attach to the core protein by O-glycosylation of a serine residue that is N-terminal to a glycine [1 – 5].

HSPGs consist of a protein core to which several linear HS chains are covalently O-linked. The polysaccharide chains are typically composed of repeating hexuronic and D-glucosamine disaccharide units that are modified at various positions by sulfation, epimerization and N-acetylation, yielding clusters of sulfated disaccharides separated by low or non-sulfated regions [1 – 4]. Glypicans are HSPGs that are linked to the cell membrane by a glycosylphosphatidylinositol anchor. Syndecans are proteoglycans that span the cytoplasmic membrane once. Syndecan glycosaminoglycans are mainly but not exclusively HSs [1].

Function

HSPGs are molecules associated with the cell surface and extracellular matrix (ECM) [1 – 5]. HS binds to ECM proteins and participates in cell-cell and cell-ECM interactions. HS chains bind a variety of proteins and growth factors, and thereby play pivotal roles in the control of normal and pathological processes [2 – 6]. HSPGs have also a co-receptor role in which the proteoglycans assemble with other cell surface molecules, resulting in a functional receptor complex [4 – 6]. Because of the multiple roles of HSPGs in cell and tissue physiology, their cleavage is likely to alter the integrity of tissues and provides a mechanism by which cells can detach from one another and respond rapidly to changes in the microenvironment of a tissue. Enzymatic degradation of HS appears therefore to be involved in essential biological processes like pregnancy, morphogenesis, inflammation, neurite outgrowth, angiogenesis, and cancer metastasis [7 – 9].

Heparin

Heparin is a linear polysaccharide produced by mast cells and composed of a polymer of alternating derivatives of D-glucosamine (N-sulfated or N-acetylated) and uronic acid (L-iduronic or D-glucuronic acid) connected by glycosidic linkages [10, 11]. Heparin is structurally related to HS but has higher N- and O-sulfate contents [10]. Commercial heparin is isolated from porcine mucosa and bovine lung, and its molecular weight ranges from 10 to 25 kDa. Heparin has an anticoagulant effect due to its ability to catalyze the inhibitory reaction between anti-thrombin and its target proteases: thrombin (factor IIa) and factor Xa (FXa) [12]. Low molecular weight heparin (LMWH) is a widely used anticoagulant; it acts through AT-mediated inhibition of FXa activity [13]. Both heparin and LMWH are used in experimental models to inhibit the enzymatic activity of heparanase [7].

Heparanase

History

Mammalian heparanase (heparanase-1) was first cloned by the groups of Vlodavsky [7] and Parish [14] in 1999 from human placenta and human hepatoma cell line (SK-hep-1). Subsequently, cloning of the same gene was reported by several groups, from human [15, 16] and other species [17–21]. McKenzie et al. reported in 2000 the cloning of a human cDNA with about 40 % homology to heparanase-1 [22]. The expression of this clone (designated heparanase-2) resulted in a protein that lacks heparan sulfate degradation ability, and whose function is still unknown. Despite earlier reports of the existence of several distinct mammalian HS-degrading endoglycosidases (heparanases), heparanase-1 is so far the only known mammalian enzyme capable of disassembling HS. Recently, we cloned several splice variants of heparanase-1 from *Spalax* (subterranean blind mole rat) and human [19, 23, and our unpublished results].

Molecular and biochemical properties

Human heparanase cDNA contains an open reading frame of 1629 bp that encodes for 543 amino acids [7]. Human heparanase is synthesized as a latent 65-kDa precursor whose processing involves proteolytic cleavage and formation of an active enzyme composed of 50-kDa subunits (Lys^{158}–Ile^{543}) associated non-covalently with an 8 kDa peptide (Gln^{36}–Glu^{109}) [24–26]. Processing and activation occur during incubation of the full-length 65-kDa recombinant pro-enzyme with several normal and transformed cells [7, 27]. Cathepsin L was implicated in activation of latent heparanase, as incubation of the recombinant 65-kDa pro-heparanase with Cathepsin L resulted in proper processing and activation of the heparanase enzyme [27]. Site-directed mutagenesis revealed that the heparanase enzyme has a catalytic mechanism that involves two conserved acidic residues, a proton donor at Glu^{225} and a nucleophile at Glu^{343} [28]. Heparanase exhibits maximal endoglycosidase activity between pH 5.0 and 6.0, and is inactivated at pH>8.0 [29]. The poorly vascularized hypoxic core of malignant tumors might provide the acidic environment required for degradation of HS by heparanase [8]. At physiological pH, little heparanase enzymatic activity is detected, but non-enzymatic functions of heparanase (e.g. leukocyte adhesion in response to inflammatory conditions) may still be preserved [29].

Following the cloning of human heparanase-1, it was cloned from chicken [18], rat [30], mouse [17], bovine [20, 21] and *Spalax* [19]. The homology to human heparanase was highest among mammalian species (especially bovine and *Spalax* heparanases), while the homology rate with chicken was about 62 % (Fig. 1).

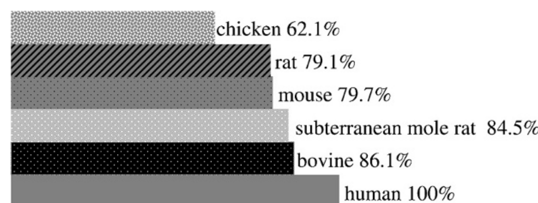

Figure 1. Homology between the cDNA sequences of human heparanase and those of bovine, *Spalax*, mouse, rat and chicken.

Based on its predicted amino acid sequence, human heparanase contains a 35-amino acid N-terminal signal peptide (Met^{1}–Ala^{35}) [7], versus 26 in *Spalax* [19] and 19 amino acids in the chicken heparanase [18]. Shorter signal peptides resulted in high secretion and cell surface localization of the heparanase enzyme [18, 31]. The 50-kDa subunit of human heparanase contains six putative *N*-glycosylation sites, while mouse, rat and chicken heparanases possess four, and *Spalax* three. Glycosylation is not required for enzymatic activity, but was implicated in the regulation of heparanase secretion [32].

Regulation

Promoter methylation. Cytosine methylation of the heparanase promoter was associated with inactivation of the affected allele. Extensively methylated CpG islands were found both in human choriocarcinoma (JAR) and rat glioma (C-6) cells which lack heparanase activity. Treatment of these cells with demethy-

lating agents (5-azacytidine, 5-aza-2'-deoxycytidine) resulted in promoter hypomethylation accompanied by reappearance of heparanase mRNA, protein and enzymatic activity [33]. Upregulation of heparanase expression and activity by demethylating drugs in C-6 rat glioma cells was associated with a marked increase in lung metastasis. The increased metastatic potential was inhibited in mice treated with laminarin sulfate, a potent inhibitor of heparanase activity [33, 34]. Promoter hypomethylation was associated with increased heparanase expression in prostate cancer tissues [35]. Methylation was significantly higher in normal bladder than in bladder cancer, and inversely correlated with heparanase expression [36]. These data indicate that methylation of the heparanase promoter plays a role in the regulation of its expression. This mode of regulation may be disturbed in malignant tissues, resulting in hypomethylation of the heparanase promoter, increased expression of the heparanase gene, and enhanced tumor metastasis and angiogenesis..

Regulation by p53. p53 is a transcription factor that regulates a wide variety of cellular promoters. Mutational inactivation of tumor suppressor p53 is found in 40–50 % of human cancers, and is the most frequent genetic alteration detected in human tumors [37]. The heparanase gene is suppressed by wild-type p53 under normal conditions. Inactivation of p53 in cancer cells results in induction of heparanase expression. Wild-type p53 binds to the heparanase promoter and inhibits its activity, whereas a mutant p53 variant fails to exert an inhibitory effect [38]. The regulation of heparanase expression by p53 may provide a possible explanation for the high levels of heparanase observed in a variety of cancer tissues, compared to healthy controls [38].

Regulation by tumor necrosis factor-alpha and interferon-gamma. Tumor necrosis factor-alpha and interferon-gamma were found to induce heparanase in cultured endothelial cells (2–3-fold increase in mRNA expression) and upregulate its enzymatic activity [39]. Both factors were associated with local heparanase induction upon elicitation of delayed-type hypersensitivity (DTH) reaction in the mouse ear. Administration of anti-heparanase small interfering RNA (siRNA) or an inhibitor of heparanase enzymatic activity effectively halted the DTH inflammatory response [39].

Regulation by estrogen. Estrogen induces heparanase mRNA transcription in estrogen receptor-positive, but not in estrogen receptor-negative, breast cancer cells. Four putative estrogen response elements were identified in the heparanase promoter region. Transcription of a luciferase reporter gene driven by the heparanase promoter was significantly increased in estrogen-receptor positive MCF-7 human breast carcinoma cells after estrogen treatment [40]. The Estrogen effect on heparanase mRNA expression levels was abolished in the presence of the pure anti-estrogen ICI 182780, indicating that the classic estrogen receptor pathway is involved in transcriptional activation of heparanase. In a mouse model, exposure to estrogen augmented levels of the heparanase protein in MCF-7 cells embedded in Matrigel plugs and correlated with increased plug vascularization [40]. Heparanase gene expression and enzymatic activity increased in endometrial cells when exposed to 17 beta-estradiol [41]. Tamoxifen stimulates heparanase transcription, conferring a proliferation advantage to breast carcinoma cells grown on extracellular matrix [42].

Regulation by glucose. Heparanase expression is upregulated in endothelial cells exposed to high glucose [43], while incubation of endothelial cells with high glucose and insulin does not enhance heparanase expression [43]. Glomerular heparanase expression is upregulated in rats and mice suffering from streptozotocin-induced diabetes [44].

Functions

Normal development and tissue remodeling. Little is known about heparanase contribution to normal cell and tissue physiology. Expression of heparanase mRNA in normal human tissues is restricted primarily to the placenta, lymphoid organs and keratinocytes [8, 9, 45]. High levels of the heparanase enzyme are also detected in blood platelets. Unlike its limited expression in human tissues, heparanase is highly expressed in diverse *Spalax* tissues [19]. Heparanase is detected in *Spalax* liver, heart, brain, and eye. Expression of heparanase in this subterranean mammal may have a role in *Spalax* adaptation to underground life, and may contribute to the increased density of blood vessels observed in some of its tissues (compared to mammals residing above ground) [19]. The enzyme appears to be involved in embryonic implantation and development [18, 45], wound repair, HS turnover, tissue remodeling, immune surveillance, and hair growth [46, 47].

Angiogenesis. HSPGs and extracellular matrix-degrading enzymes were associated with a number of angiogenesis-related cellular events (i.e., cell invasion, migration, adhesion, differentiation, and proliferation) [6, 48, 49]. Heparanase degradation of HS at

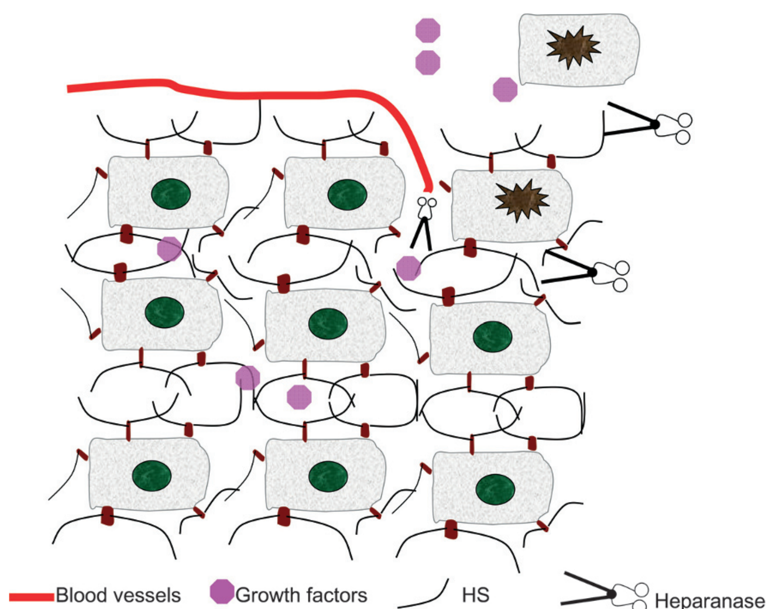

Figure 2. Heparanase degrades heparan sulfate on the cell surface and at the extracellular matrix. This activity results in releasing growth factors bound to the extracellular matrix, induces new blood vessels formation, and enhances tumor growth and metastasis.

the extracellular matrix paves the way for new blood vessels to grow inside the malignant tumor, and releases growth factors bound to the ECM (i.e., bFGF, VEGF), which further enhances tumor angiogenesis (Fig. 2). Moreover, HS fragments released by heparanase potentiate bFGF receptor binding, dimerization, and signaling [6, 49]. Heparanase participates directly in VEGF gene regulation: overexpression of heparanase in human embryonic kidney 293, MDA-MB-435 human breast carcinoma, and rat C6 glioma cells resulted in a 3- to 6-fold increase in VEGF protein and mRNA levels, which correlated with elevation of p38 phosphorylation [50]. Heparanase overexpression in U87 glioma [51], HT 29 colon carcinoma [52], MCF7 [53], human myeloma cells [54], and MDA-MB-231 breast carcinoma cells [55] correlated with enhanced xenograft tumor growth and vascularization. The level of heparanase mRNA expression was directly correlated with microvessel density in a variety of malignant tumors [56, 57].

Metastasis. Expression of heparanase in tumor cells correlates with increased metastatic potential [7–9, 58]. Heparanase cleaves HS, which results in the disassembly of subendothelial ECM and extravasation of blood-borne cancer cells (Fig. 2). High levels of heparanase were detected in urine samples of some patients with aggressive metastatic disease [59]. Overexpression of heparanase in B16 melanoma cells results in increased lung colonization by the melanoma cells [7]. *In vivo* study showed that T-lymphoma cells transfected with the heparanase gene metastasizes to the liver more than control non-transfected cells [31]. Heparin and low molecular weight heparin [60, 61], which inhibit experimental metastasis, also inhibit tumor cell heparanase [34, 60–62]. Mouse melanoma (B16-BL6) tumor cell line possess high levels of endogenous heparanase and are known to have high metastatic potential [63]. B16-BL6 transfected with anti-heparanase siRNA resulted in reduced heparanase expression and enzymatic activity. Lung colonization in a mouse experimental metastasis model was markedly (>90%) reduced by anti-heparanase siRNA compared to control cells [63]. Patients with heparanase-positive tumors exhibited a significantly higher rate of local and distant metastasis compared to patients with heparanase-negative malignancies. The postoperative survival was inversely correlated with heparanase expression [54, 64–68].

Inflammation. Heparanase is implicated in inflammation, mainly through degradation of the ECM, allowing inflammatory cells to reach their target tissue. Heparanase-inhibiting molecules inhibit T cell-mediated delayed-type hypersensitivity (DTH) [39], experimental autoimmune encephalomyelitis, and adjuvant arthritis [69, 70]. Heparanase is produced locally by the endothelium at the site of DTH-associated inflammation in response to tumor necrosis factor-alpha and interferon-gamma [39].

Heparanase non-enzymatic activities. Heparanase mediates cell adhesion regardless of its enzymatic activity [71]. Overexpressing heparanase in the anchorage-independent mouse Eb lymphoma cells resulted in their adhesion to ECM or to endothelial cell monolayers. This adhesion was not affected by

laminarin sulfate (a potent inhibitor of heparanase enzymatic activity [34]). When a mutated heparanase lacking enzymatic activity was used, enhanced cell adhesion was still observed [71].
Heparanase activates endothelial cells and elicits angiogenic responses. Addition of the 65-kDa latent heparanase to endothelial cells enhances Akt signaling. Heparanase-mediated Akt phosphorylation is independent of its enzymatic activity, or the presence of cell membrane HS proteoglycans. Heparanase also stimulates phosphatidylinositol 3-kinase-dependent endothelial cell migration and invasion [72].

Inhibition of heparanase

Heparin and low molecular weight heparin. Heparin and low molecular weight heparin were shown to inhibit HS degradation by heparanase [7]. Early studies with the platelet heparanase showed that it could cleave the glucuronide linkage in oligosaccharides containing the antithrombin (AT) binding sequence of heparin and that the cleavage products lack affinity for AT [73]. Commercial heparin and LMWH are susceptible to cleavage by heparanase *in vitro*, and this cleavage significantly neutralizes the anticoagulant properties of these polysaccharides [74]. Transgenic mice overexpressing heparanase in all tissues exhibited a shortened activated thromboplastin time (APTT) compared to control mice [74]. Degradation of heparin and LMWH by heparanase *in vivo* may be relevant in situations in which heparanase is overexpressed, and treatment with heparin or LMWH is needed (e.g., thromboembolic events in patients with malignant tumor expressing heparanase) [74, 75]. Non-anticoagulant species of heparin and various sulfated polysaccharides which inhibit experimental metastasis, also inhibited tumor cell heparanase [8, 61, 76, 77].

Suramin. Suramin, a polysulfonated naphthylurea, inhibits melanoma-derived heparanase in a dose-dependent manner [78]. Suramin had a remarkable inhibitory activity against B16 melanoma cell invasion through reconstituted basement membranes [78]. Moreover, Suramin exhibited anti-tumor growth, metastasis, and angiogenesis in several studies [79, 80].

Calcium spirulan. Calcium spirulan, isolated from a blue-green alga, *spirulina platensis*, is a sulfated polysaccharide chelating calcium and mainly composed of rhamnose. Calcium spirulan significantly inhibits degradation of heparan sulfate by purified heparanase, and reduces experimental lung metastasis when co-injected with B16-BL6 cells [81, 82]. Calcium spirulan inhibits tumor invasion by colon cancer and fibrosarcoma cells [81].

Phosphomannopentaose sulfate (PI-88). PI-88 is a sulfated oligosaccharide that inhibits heparanase activity. Animal studies demonstrated that PI-88 can inhibit tumor growth, and reduce tumor metastasis and vascularity [62]. Beside its anti-neoplastic properties, PI-88 possesses anticoagulant effects (it elongates activated partial thromboplastin time). Phase I studies identified thrombocytopenia and pulmonary embolism as dose-limiting toxicities [83, 84]. Clinical trials are being performed to define the role of PI-88 in cancer therapy.

Maltohexaose sulfate. Maltohexaose sulfate was reported to have both antiangiogenic and heparanase inhibitory properties [77].

Laminarin sulfate. Laminarin sulfate is a polysulfated polysaccharide isolated from the cell walls of seaweed and subjected to chemical sulfation [85]. Laminarin sulfate is a potent inhibitor of ECM degradation by mammalian heparanase [85]. Heparanase activity expressed by B16-BL6 mouse melanoma cells and 13762 MAT rat mammary adenocarcinoma cells was inhibited by laminarin sulfate, but there was no inhibition by sodium laminarin [34]. Animal studies have shown that laminarin sulfate inhibits experimental autoimmune encephalomyelitis [85]. Tumor metastasis from B16-BL6 melanoma and 13762 MAT rat mammary adenocarcinoma cells were markedly inhibited by laminarin sulfate [34].

Anti-heparanase antibodies. Since the cloning of human heparanase, several anti-heparanase antibodies were described. Zetser et al. described antibody #733, which was raised against the peptide ^{158}KKFKNSTYRSSSVD^{171} of human heparanase. Antibody #733 preferentially recognizes the 50-kDa subunit of heparanase, compared to the latent 65-kDa heparanase precursor. *In vitro* studies showed that antibody #733 partially neutralizes the enzymatic activity of heparanase [86]. Myler et al. described another anti-heparanase antibody that was tested *in vivo* in a rat carotid balloon injury model. This antibody was effective in suppressing heparanase activity, inhibited bFGF release, and correlated with significant reduction in neointima formation [87].
Interestingly, Gingis-Velitski et al. reported the production of a monoclonal anti-heparanase antibody (6F8) that enhances the enzymatic activity of recombinant heparanase, facilitates *in vitro* tumor cell invasion, and improves wound healing in a mouse punch model [88].

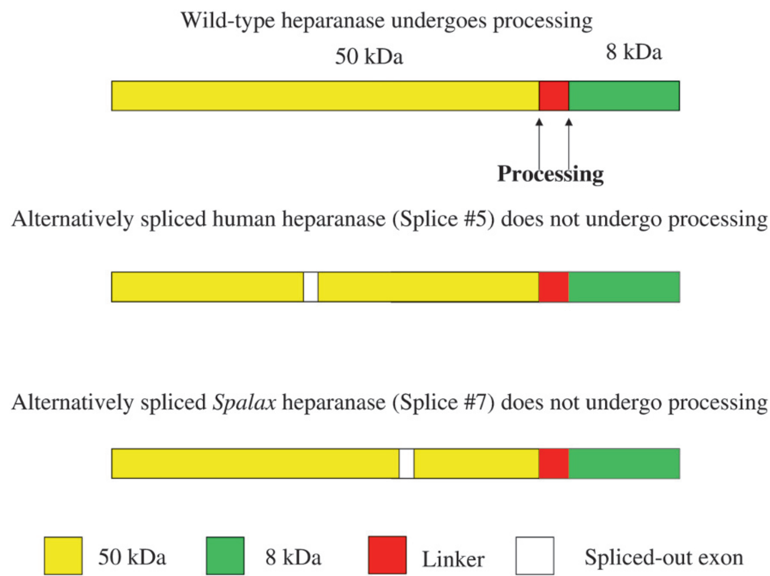

Figure 3. Human wild-type heparanase is synthesized as a latent 65-kDa precursor. Processing by proteolytic cleavage results in formation of an active enzyme, composed of a 50-kDa subunit associated non-covalently with an 8-kDa peptide (top). Splice variant #5 (middle) lacks exon #5, and splice variant #7 results from skipping of exon #7 (bottom). Both splice variants lack intrinsic heparan sulfate degradation ability.

Peptides inhibiting heparanase activity. Levy-Adam et al. characterized a heparin binding domain at the N-terminus of the 50-kDa active heparanase subunit of the heparanase protein (amino acids $Lys^{158}-Asp^{171}$). A peptide composed of these amino acids (designated KKDC) inhibited heparanase enzymatic activity *in vitro* in a dose-dependent manner. The KKDC peptide was shown to interfere with the binding of heparanase to its HS substrate [89].

Heparanase gene silencing. Heparanase gene silencing was tested in experimental models as a method to suppress heparanase-related tumor metastasis and angiogenesis. Edovitsky et al. demonstrated that anti-heparanase ribozyme- or siRNA-mediated gene silencing results in down-regulation of heparanase enzymatic activity, tumor invasiveness, angiogenesis, and metastasis [63]. Zhang et al. reported that silencing of heparanase in breast cancer cells results in reduced invasion and adhesion *in vitro* [90]. In an MDA-MB-435 cell xenograft model, tumors treated with siRNA were less vascularized and less metastatic than control cells [90]. In another report, the expression of heparanase in human hepatocellular carcinoma cells was suppressed by antisense oligodeoxynucleotide or by RNA interferencing. This resulted in reduction of heparanase expression, leading to inhibition of invasiveness, metastasis, and angiogenesis of these cells, both *in vitro* and *in vivo* [91].

Oligomannurarate sulfate (JG3). Oligomannurate sulfate (JG3) is a semisynthetic sulfated oligosaccharide derived from marine oligomannurarate blocks. JG3 inhibits heparanase enzymatic activity via binding to heparin binding domain on heparanase. JG3 abolishes heparanase-induced invasion, inhibits the release of heparan sulfate-sequestered basic fibroblast growth factor from the extracellular matrix, and suppresses subsequent angiogenesis. JG3 decreased lung metastasis of B16-F10 melanoma cells in a dose-dependent manner [92].

Alternative splicing of heparanase

On average, each human gene generates three alternatively spliced mRNAs [93–97]. This takes place by several mechanisms, including exon skipping, skipping part of an exon, or intron retention [93–98]. The genomic organization of human heparanase was described by Dong et al. [99]. They also described a splice variant in the untranslated regions of the gene. Recently, we described alternative splicing in the coding region of heparanase both in human [23] and *Spalax* [19] (Fig. 3). Alternatively spliced human heparanase was cloned from kidney tissue of a patient suffering from renal cell carcinoma. This splice variant lacks exon #5 and is devoid of heparan sulfate degradation ability. Unlike wild-type heparanase, which in latent form is detected in cell culture incubation medium, splice #5 was not detected in the medium, and seemingly is not secreted. The function of splice #5 and its roles in physiology and disease are still unknown. Alternatively spliced *Spalax* heparanase was cloned from the kidney of healthy animals and was detected later in a variety of tissues [19]. This splice variant lacks exon #7, does not undergo proteolytic cleavage (compared to the wild type heparanase), and is not detected in the incubation medium. Other splice variants of heparanase were detected and are currently under investigation [our unpublished data].

Heparanase two

Mckenzie et al. reported the cloning of a cDNA encoding a human protein designated heparanase 2 (HPA2) [22]. HPA2 possesses about 40% amino acid similarity compared to heparanase 1. Alternative splicing of HPA2 yields three different mRNAs, encoding putative proteins of 480, 534, and 592 amino acids. HPA2 exhibits markedly different mRNA distribution relative to HPA1 in both normal and cancer tissues. The function of HPA2 in physiological and pathological conditions is still undefined.

Conclusions

Heparanase is an endoglycosidase that functions in a variety of physiological and pathological conditions. The role of heparanase in normal development is still not well characterized. The fact that transgenic mice overexpressing heparanase in all tissues are viable and fertile suggests that heparanase may have a variety of physiological functions [100]. These mice have stronger bone tissue, grow hair faster than controls, and have special wound-healing characteristics [100]. Heparanase knockout mice are expected to unfold the functions of heparanase in embryogenesis and normal development. The role of heparanase in cancer progression, growth, metastasis, and angiogenesis is much more defined than its role in physiology. Studies that have been performed in the last 3 decades suggest heparanase as a target for anticancer therapy. Basic research resulted in the characterization of several inhibitors of heparanase, of which PI-88 is now being tested in clinical trails. Success in these clinical trails will accelerate the efforts for finding efficient, non-toxic medications capable of suppressing heparanase tumorigenic activity. Beside its enzymatic function in HS degradation, heparanase possesses a variety of non-enzymatic functions, such as cell adhesion and signaling. Alternatively spliced forms of heparanase seem to have important roles in regulating of the wild-type enzyme [our unpublished data]. Altogether, it seems that heparanase and its spliced forms will continue to be the subject of intensive research in the coming years.

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