MOLECULAR STRUCTURE OF HEPARAN SULFATE FROM SPALAX: IMPLICATIONS OF HEPARANASE AND HYPOXIA

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Summary

Spalax, a subterranean blind mole rat, is well adapted to live in an extreme hypoxic environment through upregulated expression of growth factors and enzymes for ensuring sufficient oxygen supply. One of the overexpressed enzymes is heparanase, an endoglucuronidase that selectively cleaves heparan sulfate (HS) and is implicated in angiogenesis. To assess the implications of the heparanase in Spalax, we have characterized the structure of HS isolated from various organs of the animal. The oligosaccharides obtained after deaminative cleavage of HS samples from the tissues show an overall higher sulfation degree, distinct from that of murine tissues. Of particular significance was the appearance of a trisaccharide moiety in the tissues examined, apart from the even numbered oligosaccharide fractions typically found in HS from human and mouse tissues. The formation of this odd-numbered saccharide is a consequence of heparanase action, in agreement with the notion of high expression of the enzyme in this species. Analysis of HS extracted from human embryonic kidney cells (HEK293) after exposure to hypoxic condition revealed a structural change in the distribution of oligosaccharides similar to HS derived from Spalax organs. The alterations are likely due to upregulated activity of heparanase, as real-time RT-PCR showed a 2-fold increase in heparanase mRNA expression in the hypoxia treated cells. HEK293 cells stably overexpressing Spalax heparanase produced HS sharing similarity with that from the Spalax organs, and exhibited enhanced MAPK activity in comparison with HEK293 cells, indicating a regulation role of the heparanase in the activity of growth factors.

Introduction

The blind subterranean mole rat Spalax ehrenbergi is a fossorial mammal which spends its entire life in sealed underground tunnels, where the atmosphere can be severely hypoxic/hypercapnic (1,2). To survive in such hostile environment the animal has developed several strategies such as structural adaptations in tissues to ensure an efficient oxygen delivery/uptake (3), and mutation in the tumor suppressor gene P53 to favor transcription of cell cycle arrest and DNA repair genes versus
apoptosis genes (4). Moreover, a higher expression level of genes such as hypoxia-inducible factor-1α (HIF-1α), a key factor in inducing genes facilitating adaptation and survival of cells under hypoxia (5), and erythropoietin (Epo), was observed (6). An atypical expression pattern of the vascular endothelial growth factor (VEGF) leading to an enhanced blood vessels density has also been found in Spalax (7,8).

Interestingly, heparanase, an endo-β-D-glucuronidase specifically cleaving HS, was found expressed at high levels in diverse Spalax tissues (9). Though the established biochemical function of heparanase is to catalyze the degradation of HS, this degrading property of heparanase regulates several processes of physiological and pathological importance. Indeed this enzyme plays significant roles in morphogenesis and development as well as in matrix remodeling (10,11) and was shown to be involved in cell invasion associated with angiogenesis, inflammation and cancer metastasis (12-15). These functions of heparanase are largely linked to the modulation of molecular structure of HS (16).

HS is a polysaccharide attached to a protein core found on the cell surface and in the extracellular matrix (ECM) of all mammalian tissues. This negatively charged macromolecule has the potential to interact with a wide array of physiologically active ligands and is thereby involved in a variety of normal and pathophysiological processes (17). The diverse functions of HS are devoted to its heterogeneous property of molecular structure, generated by a highly regulated biosynthesis process. The biosynthesis of HS is a complex mechanism, initiated by the formation of a backbone composed of alternating disaccharides units containing D-glucuronic acid (GlcA) and D-glucosamine (GlcNAc). This polymer undergoes modifications, e.g. N-deacetylation and N-sulfation of the GlcNAc unit, C5-epimerization of GlcA into L-iduronic acid (IdoA) and multiple O-sulfation at various positions (18). As all the sugar residues are not identically modified, the HS molecule is typically organized in domains of variable sulfation degree, comprising N-acetylated and N-sulfated regions (NA and NS-domains) interrupted by regions composed of alternating N-acetylated and N-sulfated disaccharide units (NA/NS-domains) (19,20). Thus, the biosynthesis process generates a highly heterogeneous pool of HS polymers that nonetheless appear to be cell/tissue specific as the result of a strict control of the biosynthetic machinery (21).

We report the molecular structure of HS from various Spalax organs and implications of hypoxia and heparanase upon HS structure. The pattern of HS from Spalax is tissue specific, and associated with an overall highly sulfated structure, which may be tailored by heparanase and hypoxia. Unexpectedly, analysis of deamination cleavage products of HS samples from Spalax organs detected a trisaccharide fraction that has not been observed in murine HS samples. To explore the correlation between hypoxia, heparanase and HS structure, we examined HS from HEK293 cells that stably expresses Spalax heparanase in comparison with mock transfected cells and found that overexpression of Spalax heparanase in HEK293 cells resulted in a HS structure similar to that of HS extracted from the Spalax organs, while overexpression of human heparanase in the same cells did not produce the trisaccharide fraction, suggesting a different enzymatic action of two heparanase species. The finding of trisaccharide moiety in the HS degradation products provides additional information on substrate recognition property of heparanase. The effect of Spalax heparanase on cellular activity was evaluated by measuring Erk phosphorylation in MAPK signaling pathway.

Experimental Procedures

**Animals and Animal care-** Spalax female animals (100-150 g) were captured in the field and kept in animal facilities (Haifa, Israel) for at least 3 months before use. Animals were housed in individual cages under controlled conditions at 22 to 24°C and fed with carrots and apples. In this study, two animals were used, one belongs to the species Spalax judaei (S60) that has 60 chromosomes, and the other is Spalax galili (S52) that has 52 chromosomes. The experiment was approved by the ethic committee of the University of Haifa, Israel. All the presented data are from Spalax judaei (S60) if not stated otherwise.

**Metabolic labeling and dissection of organs** - Animals were metabolically labeled with [3H]glucosamine for 6 h followed by terminal injection with Ketaset CIII (Fort Dodge Animal Health, Fort Dodge, IA) at 5 mg/kg of body weight. The organs were dissected, immediately
frozen in liquid nitrogen and stored at -80°C prior to use.

Cell culture and hypoxia treatment- HEK293 cells, transfected with cDNAs coding for Spalax or human heparanase, or a mock vector (9) were grown in DMEM (Invitrogen) supplemented with 10% fetal calf serum and penicillin/streptomycin (60 and 50 µg/ml, respectively). The cells were cultured at 37°C in humidified air containing 5% CO₂ and 21% O₂. Hypoxia was generated in a humidified chamber that was flushed with 95% N₂ and 5% CO₂ gas mixture until 1% O₂ was reached and then cultured at 37°C for 24 h. Equal number of cells were used in each experiment for hypoxia and normoxia cultures and there were no differences in cell viability under these conditions as determined by trypan blue viability test.

Purification of HS from organs- The frozen organs were cut into small pieces and subjected to protease (Sigma) digestion (2 U per 0.02 g dry weight sample in 50 mM Tris-HCl pH 7.4, 1 mM CaCl₂, 1% Triton X-100) for 24 h at 56°C. The proteolyzed homogenates were boiled (5 min) to deactivate the protease and subjected to benzonase (Merck) digestion (12.5 U per 2 g tissue) at 37°C for 2 h. After centrifugation at 3500 g for 10 min, the supernatants were recovered and applied to a 2 ml DEAE-Sephacel column (GE Healthcare Biosciences) equilibrated in 50 mM Tris-HCl pH 7.4, 0.25 M NaCl. The column was first washed with 20 ml of the equilibration buffer and then further washed with 0.25 M NaCl in 50 mM NaAc buffer, pH 4.5, until no radioactivity was detectable in the flow through. The bound material was eluted with 2 M NaCl in 50 mM NaAc buffer, pH 4.5. The eluted material was pooled and desalted on a PD-10 column (GE Healthcare Biosciences) and lyophilized. The materials were digested with chondroitinase ABC (Seikagaku; 0.2 U in 50 mM Tris-HCl, pH 8) for 24 h at 37°C. After heat inactivation of the enzyme, the HS was isolated on a 0.5 ml DEAE-Sephacel column using the same procedure as described above. The purified HS samples were lyophilized and stored at -20°C.

Analysis of HS structure- The molecular size of HS was analyzed by gel chromatography on a Superose 12 column (GE Healthcare Biosciences). For fine structural analysis, HS from most Spalax organs were first reduced by NaBH₄ then subjected to deaminative cleavage with HNO₂ at pH 1.5 (22), followed by reduction with NaBH₄ to chemically label the oligosaccharides at the reducing-end. In liver and kidney, higher level of [³H]glucosamine was incorporated during the metabolic labeling process, hence the HS samples were reduced with cold NaBH₄ after deaminative cleavage. For analysis of N-sulfation pattern of the HS chains, one portion of the deamination cleavage products was size separated by gel chromatography on a Bio-Gel P10 column (1 x 200 cm) (BIO-RAD) in 0.5 M NaCl. The rest of the sample was fractionated by gel chromatography on a Sephadex G-15 column (1 x 180 cm) (GE Healthcare Biosciences) equilibrated with 0.2 M NH₄HCO₃ to recover the disaccharides. The disaccharides were analyzed on a Partisil-10 SAX HPLC column (Whatman Inc.) eluted with increasing concentration of KH₂PO₄ (0.01-0.4 M) (23).

Metabolic labeling and isolation of HS from cells- HEK293 cells were grown in 75 cm² flasks to 90% confluence, then changed to fresh medium containing 800 µCi [³H]glucosamine and cultured for 24 h. The cells were harvested and the media were collected for isolation of HS. The cells were lysed by incubation in a lysis buffer (4 M Urea, 1% Triton, 50 mM Tris-HCl pH 7.4, 0.25 M NaCl) at 4°C for 1 h, followed by alkali treatment with 0.5 M NaOH at 4°C overnight. After neutralization with 0.5 M HCl, HS chains were purified and analyzed as described for purification of HS from organs.

Isolation and analysis of HS trisaccharide- The trisaccharide fraction was isolated from three HS samples, from the liver and kidney of Spalax judaei (S60), the brain of Spalax galili (S52) and Spalax transfected HEK293 cells. The HS samples were subjected to deaminative cleavage and the resulted oligosaccharide products were separated on a Sephadex column. Fractions corresponding to the elution position of trisaccharide (the column was calibrated with di- and tetra-saccharides) were pooled and subjected to treatment with either β-glucuronidase (350 U) (Sigma Aldrich), or a combination of β-glucuronidase, α-iduronidase (1.19 µg) and iduronidase-2-O-sulfatase (0.39 µg) (kindly provided by Dr. John Hopwood, Adelaide, Australia) in 50 mM NaAc pH 4.5, at
37°C for 24 h. The digested samples were analyzed on the same Sephadex column. In an alternative approach the trisaccharide was degraded by chemical means. The trisaccharide species from the brain, retrieved from a Bio-Gel P10 column after deaminative cleavage at pH 1.5, were lyophilized and dissolved in 1 ml of hydrazine hydrate (Fluka; H₂O content, 36%) containing 1% (w/v) hydrazine sulfate and heated in sealed glass tubes at 96 °C for 4 h. The samples were repeatedly evaporated to dryness and desalted on a PD-10 column (GE Healthcare Biosciences) followed by deaminative cleavage at pH 3.9 (cleaving at N-unsubstituted GlcN residues; see Scheme 1). The resulted product was reduced with NaBH₄ and analyzed on the Sephadex column.

Quantification of gene expression- Cells were grown in normoxia (21% O₂) or hypoxia (1% O₂) conditions for 24 h and the total RNA was extracted using the RNeasy Mini Kit (QIAGEN) according to the manufacturer’s instructions. Synthesis of cDNA was carried out using 2 μg of total RNA with the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer’s instructions. cDNA from Spalax kidney was prepared as described previously (9). Two cDNA preparations of Spalax originating from two different animals were used. cDNA from kidney of a C57Bl mouse was prepared as for the cDNA from cells. Quantitative real-time RT-PCR was performed using iQ™ SYBR Green Supermix (BIO-RAD) in a Mini-Opticon (BIO-RAD), according to the standard protocol of the manufacturer, with optimized annealing temperature for different primers. The experiments were performed in triplicates by two independent real-time PCR measurements, except for the N-deacetylace/N-sulfotransferase-1 (NDST-1) expression in Spalax that was mean values of two different animals analyzed in duplicates. All gene expression was normalized against the housekeeping gene β-actin. The primers were designed to be located on different exons using OligoPerfect™Designer (Invitrogen). Human heparanase primers (Forward: “GGTTCCTCATCCTCCTGGG” Reverse: “TGACTTGAGATGGCCAGTAACTTC”) annealed at 58°C, human β-actin primers (Forward: “GAGCTACGAAGCTGCTGACG” Reverse: “GTAGTTTGCTGGATGCCACAG”) annealed at 60°C, human VEGF-A primers (Forward: “AGGAGGAGGGCAGAATCATCA” Reverse: “CTCGATTGGATGCGAGTAGCT” annealed at 60°C and human/mouse NDST-1 primers (Forward: “CCACAACTATCACAAGGCGATCG” Reverse: “GAAAGGTGTACTTTAGGGCCAC” annealed at 60°C.

Growth factor-HS interactions- The interaction of HS and growth factor was assayed using a nitrocellulose binding method as described earlier (24). Briefly recombinant fibroblast growth factor-2 (FGF-2) (PeproTech) was incubated with metabolically [¹⁴C]glucosamine labeled HS isolated from mock-HEK293 cultured in normoxia or hypoxia. HS was mixed with 50 ng FGF-2 in 200 μl of PBS pH 7.4 and incubated at room temperature for 2 h. The mixture was passed through a nitrocellulose filter (Sartorius, diameter 25 mm, pore size 0.45μm), followed by washing with 10 ml of PBS. The HS trapped on the filter in a complex form with FGF-2 was released by addition of 2 M NaCl and measured by scintillation counting.

Western blot analysis of phosphorylated Erk1/2-Cells were seeded on 6-well plates at a density of 2 × 10⁵ cells/well in DMEM medium supplemented with 10% FCS and antibiotics for 24 hours. The cells were then maintained either in starvation medium (DMEM) or in medium fortified with 10% FCS for 24 hours. In some experiments, serum starved cells were stimulated with 6 ng/ml FGF-2 (PeproTech). The medium was removed and cells were washed with PBS twice before being lysed in 100 μl of sample buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM Na₃VO₄, 1X protease inhibitor cocktail from Roche). After incubation on ice for 30 min the lysates were centrifuged for 15 min at 13,000 rpm and the supernatants were collected for protein determination. Samples of 20 μg total protein were separated by electrophoresis on SDS-PAGE (12%) and electroblotted onto a PVDF membrane (Millipore). The membrane was probed using primary antibodies against anti-phospho-Erk1/2 (Thr202/Tyr204) or endogenous level of total Erk1/2 and secondary anti-rabbit horseradish peroxidase-linked antibody (Cell Signaling). The signals were developed using an
ECL plus Western Blotting Detection System (Amersham™ GE Healthcare) and exposed to Fuji films. The results were analyzed with ImageJ (National Institutes of Health, USA).

Results

Domain organization of HS derived from Spalax organs. Metabolically radiolabeled HS samples were isolated from Spalax organs as described in “Experimental procedures”. Selected samples from brain, liver and kidney were analyzed on a Superose 12 column for assessment of chain length (Supplementary Fig. 1A) and appeared averagely shorter than the ones from corresponding murine organs (Supplementary Fig. 1B), at the exception of the brain sample. To characterize the N-sulfation pattern, HS samples from brain, heart, kidney, lung, muscle and liver were subjected to deaminative cleavage at pH 1.5, a chemical reaction cleaving the HS chains at N-sulfated glucosamine residues (Scheme 1). After cleavage, the resultant oligosaccharides were reduced with NaBH₄ (except the samples from liver and kidney which were reduced with cold NaBH₄) and analyzed on a Bio-Gel P10 column as described under “Experimental Procedures”. As an example, Fig. 1A shows the distribution of oligosaccharides produced by deaminative cleavage of HS from Spalax brain. The chromatogram pattern shows a high proportion of di- and tetra-saccharides. Notably, an additional peak between di- and tetra-saccharides was found (indicated by arrow). Gel chromatography analyses of HS samples from other organs displayed similar distribution patterns as summarized in Fig. 1B.

Assessment of disaccharide composition in NS domain of HS from Spalax organs. The high proportions of di- and tetra-saccharides in HS suggested a high degree of N-sulfation (see Scheme 1). To examine the O-sulfation pattern, the disaccharide fraction (see Fig. 1A) was pooled and analyzed by strong anion exchange chromatography. Fig. 2A depicts the analysis of disaccharides recovered from lung HS, showing the five components of disaccharides commonly found in human and murine organs. However, analysis of disaccharide samples from other organs revealed varied elution patterns. Quantification of the peak areas from each analysis indicated a distinct distribution of disaccharide species (Fig. 2B). All samples, with the exception of liver HS, contained a high proportion of the IdoA(2-OSO₃)-aManR moiety (representing an -IdoA2S-GlcNS- in the intact NS-domains). Notably, the lung contained as much as 80% of this disaccharide component, while liver HS is composed predominantly (~60%) of tri-sulfated disaccharide (representing an -IdoA2S-GlcNS6S- in the intact NS-domains). To confirm that these structural alterations of HS are not a bias of the Spalax judaei (S60), we have performed the same analysis for HS samples isolated from the specie Spalax galili (S52). The results showed essential identical organ specific patterns of N- and O-sulfation of HS from the two animals (Supplementary Fig. 2).

Effect of hypoxia and heparanase on HS structure. To investigate the correlation between hypoxia, heparanase and HS structure, we have analyzed HS isolated from HEK293 cells stably overexpressing the full-length Spalax heparanase gene. The heparanase overexpressing cells (Hpa-HEK293) and mock transfected cells (mock-HEK293) were cultured under normoxic (21% O₂) and hypoxic (1% O₂) conditions in the presence of [³H]glucosamine for 24 h. HS from both cell and medium fractions were isolated for examination of chain length by gel chromatography, as described in “Experimental Procedures”. Under normoxia conditions, HS derived from Hpa-HEK293 cell fraction was slightly more degraded than that from the mock transfected cells (Fig. 3A), while the HS chains from the conditioned medium of Hpa-HEK293 were considerably shorter than those from the mock cell culture medium (Fig. 3B). The hypoxic treatment of the cells had apparently no effect on the chain length of HS from both mock and Hpa-HEK293 cells (Fig. 3C).

To further analyze whether hypoxia treatment had any effect on the compositional structure of HS, the samples used for chain length analysis were cleaved by deaminative treatment at pH 1.5 (Scheme 1) and the resulted products were analyzed on a Bio-Gel P10 column. Interestingly, the HS from mock-HEK293 cells had exclusively even numbered oligosaccharides (Fig. 4A), while the same cells cultured under hypoxia conditions clearly displayed extra peaks eluted at the positions of tri- and penta-saccharides (Fig. 4B). Similarly, HS sample from Hpa-HEK293 cells contained...
substantial amounts of the trisaccharide (Fig. 4C). Hypoxic treatment of the heparanase overexpressing cells did not further affect the structure of HS (Fig. 4D), presumably because of an already high level of heparanase expression in these cells.

Characterization of the trisaccharide. HS samples isolated from Spalax liver and kidney were subjected to deaminative cleavage at pH 1.5, followed by reduction with NaBH₄ and analysis on a Bio-Gel P10 column. The distribution patterns of the resulting oligosaccharides produced by deaminative cleavage of metabolically [³H]glucosamine labeled HS from Spalax liver and kidney are shown in Fig. 5A and B. The chromatogram pattern resembles that of ³H-end-labeled oligosaccharides from the brain shown in Fig 1A, with a high proportion of di- and tetra-saccharides (the difference in relative proportions of variously sized oligosaccharides reflects the different labeling protocols, resulting in ³H-distribution throughout the saccharides in Fig. 3A and B, and at the reducing termini only in Fig. 1A). To characterize the material eluted at the trisaccharide position (Fig. 5A, B), the total HS from liver and kidney was incubated with NaB³H₄ to label the GlcA present at reducing end, which results from heparanase cleavage of intact HS chains prior to treatment with HNO₂ at pH 1.5 (see Scheme 1). The deaminative products were applied to a Sephadex column for preparative separation. The fractions eluted at the position corresponding to trisaccharide were collected and subjected to treatment with a mixture of β-glucuronidase, α-iduronidase and iduronidate-2-O-sulfatase. The resulted product was reanalyzed on the same column, as shown in Fig. 5C. The multiple enzyme treatment resulted in a complete shift of the peak eluted at the position of trisaccharide towards the elution position of a disaccharide. To further verify the property of trisaccharide produced in Spalax organs, material corresponding to the trisaccharide peak as indicated in Fig. 1A was isolated from the brain of Spalax galili (S52) and treated by deacetylation followed by deamination cleavage with nitrous acid at pH 3.9 (Scheme 1). The trisaccharide peak, as analyzed on the same column, is completely shifted towards the elution position of a disaccharide (Fig. 5D), indicating that the glucosamine residue in the trisaccharide is N-acetylated (the additional broad peak eluted at 90-100 ml likely represents released monosaccharides along with free-isotope). As the trisaccharide moiety was also observed in the HS from HEK293 cells overexpressing Spalax heparanase (Fig. 4C), we collected this material and treated with β-glucuronidase. Analysis of the product on the same column showed a partial shift of the peak (around 55%), suggesting a distribution of GlcA and IdoA at reducing end of the trisaccharide is about 55% and 45%, respectively (Fig. 5E).

Implication of hypoxia and heparanase on gene expression. Although the hypoxic treatment did not show an obvious effect on the chain length of HS from mock- and Hpa-HEK293 cells (Fig. 3C), the appearance of tri- and penta-saccharides in mock-HEK293 cells cultured under hypoxia conditions suggests an extra-degradation of HS (Fig.4B). Thus, we examined the expression of heparanase gene in mock-HEK293 cells cultured under normoxia and hypoxia conditions. Indeed, real-time RT-PCR showed a 2-fold increase in expression of the heparanase gene upon hypoxic treatment (Fig. 6). Further we investigated whether hypoxia treatment of the HEK293 cells had affected expression of growth factors and HS biosynthesis enzymes. As expected, real-time RT-PCR revealed a 8-fold upregulation in VEGF expression, but essentially no change in NDST-1 expression (Fig.6); while none of these genes were affected by overexpression of Spalax heparanase in HEK293 cells (Fig.6). Finally, we compared expression of NDST-1 in kidney from Spalax and mouse by real-time RT-PCR and found the Spalax had a slightly lower level of NDST-1 expression (Fig.6).

Impact of Spalax heparanase on MAPK activity in HEK293 cells. The remaining question is the functional correlation of Spalax heparanase and growth factor mediated cellular activity. To address this question, we examined Erk phosphorylation in the mock-HEK293 and Hpa-HEK293 cells cultured under different conditions. Western blot analysis revealed that overexpression of the Spalax heparanase in HEK293 cells enhanced Erk phosphorylation under starvation conditions (Fig. 7), but had no apparent effect when the cells were maintained in DMEM fortified with 10% FCS, or at DMEM supplemented with FGF-2 (data not shown).

Discussion
The biological and pathological roles of HS, generally ascribed to its interaction with proteins (17,25) are dependent on the structure of HS chains, more precisely on its sulfation patterns (18). The diversity of HS structure is by large the result of a regulated biosynthesis, however, two additional enzymes, endosulfatases (known as Sulfs and heparanase, play important roles in regulation of HS structure post-synthesis (16,26). The finding that Spalax expresses a high level of heparanase (9) led us to investigate the molecular structure of HS in this animal. The natural heparanase overexpression in Spalax provides a unique model to study the role of heparanase in remodeling of HS structure. To get an overview of HS structure in Spalax, we analyzed HS from brain, heart, liver, kidney, lung and muscles as these tissues normally require more oxygen for maintaining normal biological functions.

During HS biosynthesis, N-deacetylation and N-sulfation is a critical step for the subsequent modification reactions, as the epimerization and O-sulfation reactions can essentially only take place in the N-sulfated regions, with some exceptions (27). Consequently, the N-sulfation pattern is of primary interest in dissecting HS molecular structure. Deaminative cleavage at pH 1.5 selectively cleaves the N-sulfated glucosamine residues (Scheme 1), generating disaccharides from NS-domains, tetrasaccharides from NA/NS domains and >4-mer from NA domains. The high content of di- and tetra-saccharides in HS from Spalax (Fig. 1; Supplementary Fig. 2A) implicates an overall high degree of N-sulfation. Interestingly, the major enzyme responsible for N-sulfation (NDST-1) is neither upregulated in Spalax nor in hypoxia treated HEK293 cells (Fig. 6). This is in agreement with results obtained applying heparanase transgenic mice and tissues with upregulated expression of heparanase (16), demonstrating that there is a regulatory mechanism for enzymatic activities at the protein level. As previously observed for the disaccharide composition of NS-domains derived from various murine organs (21,28), a tissue specific disaccharide pattern was also observed in the Spalax organs examined (Fig. 2, Supplementary Fig. 2B). A high level of 2-O-sulfated disaccharides (-IdoA2S-GlcNS-) was found in lung compared to kidney, similar to the corresponding murine organs (21). The level of tri-sulfated disaccharide moiety in liver (-IdoA2S-GlcNS6OS-) ISMS in Fig. 2B) was 2-fold higher than that determined in murine liver (16).

Of particular significance is the presence of odd-numbered saccharides in HS extracted from the Spalax organs (Fig. 1, 5A and B). These components are also present in other Spalax organs analyzed (data not shown). Occurrence of the trisaccharide moiety in the deamination product of HS is assumed to be the result of heparanase action on the HS chain (Scheme 1). To find out whether appearance of the trisaccharide is a unique characteristic of Spalax organs, we analyzed HS from HEK293 cells that either stably overexpressing Spalax heparanase (Fig. 4C), or exposed to hypoxia (Fig. 4B). The distinguished peaks in the elution position corresponding to trisaccharide clearly demonstrate the consequence of extensive heparanase action in the Hpa-HEK293 cells and in hypoxia treated mock-HEK293 cells.

To verify the nature of the component eluted at the trisaccharide position, the corresponding fraction was isolated from three different sources, e.g. HS from the liver and kidney of Spalax judaei (S60) and the brain of Spalax galili (S52) as well as Spalax heparanase transfected cells. The three preparations have been characterized by three approaches. Treatment of the liver and kidney samples by a mixture of exoglycosidases (iduronidate-2-O-sulfatase, α-iduronidase and β-glucuronidase) resulted in complete shift of the trisaccharide (Fig. 5C), indicating complete removal of the non-reducing end hexuronic acid and resulting in disaccharide. While the chemical deamination cleavage in combination with deacetylation of the sample also resulted in complete shift of the trisaccharide peak (Fig. 5D), demonstrating the property of N-acetylated glucosamine residue in the trisaccharide. Finally to assess the distribution of GlcA and IdoA at non-reducing end hexuronic acid and resulting in disaccharide. While the chemical deamination cleavage in combination with deacetylation of the sample also resulted in complete shift of the trisaccharide peak (Fig. 5D), demonstrating the property of N-acetylated glucosamine residue in the trisaccharide. Finally to assess the distribution of GlcA and IdoA at non-reducing end hexuronic acid and resulting in disaccharide. While the chemical deamination cleavage in combination with deacetylation of the sample also resulted in complete shift of the trisaccharide peak (Fig. 5D), demonstrating the property of N-acetylated glucosamine residue in the trisaccharide. Finally to assess the distribution of GlcA and IdoA at non-reducing end hexuronic acid and resulting in disaccharide. 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provide a useful tool to illustrate heparanase cleavage specificity. It is interesting that Spalax heparanase produced fragments often have a GlcNAc residue at non-reducing end of the cleaved GlcA-GlcNS bound, accordingly resulted in trisaccharide (and pentasaccharide) after deamination cleavage at pH 1.5. This seems not the case with human heparanase, as analysis of HS from HEK293 cells stably overexpressing human heparanase failed to detect the trisaccharide fraction (Supplementary Fig. 3). Lacking of the trisaccharide moiety in deamination products of HS has also been observed in our previous study on a transgenic mouse overexpressing human heparanase (16). These results may suggest that human heparanase prefers a N-sulfated glucosamine, while Spalax heparanase prefers a GlcNAc, at the non-reducing end of the cleavage site. Yet, it should be noted that hypoxia treatment of HEK293 cells also resulted in production of trisaccharide (Fig. 4B), which is presumably by action of upregulated endogenous human heparanase. Whether different properties towards substrate preference are associated with the enzymes by transfected overexpression and upregulated expression of endogenous product remains to be illustrated. Moreover, it is possible that the Spalax heparanase has a preferential cleavage towards the reducing end of HS chains, since non-reducing end sequence is often highly sulfated (32) and hence is a poor substrate for Spalax heparanase. This cleavage, instead of generating small fragments like the human heparanase (16), releases mainly longer HS fragments that maybe of functional importance for the animal.

Augmented heparanase activity was previously noted in response to hypoxia (33), providing the first evidence showing the regulation effect of hypoxia on heparanase expression. The aptitude of Spalax to deal with hypoxic stress and finding of the unique structure of HS in the animal drew our attention to examine expression of heparanase in cell cultured under hypoxia condition. Real-time RT-PCR revealed a 2-fold increase in heparanase mRNA expression in response to hypoxia treatment of HEK293 cells (Fig. 6). Accordingly, analysis of HS isolated from the hypoxia treated cells resulted in a structural pattern that resembles the one from HEK293 cells overexpressing Spalax heparanase (Fig. 4B, C), suggesting a correlation of hypoxia treatment and heparanase expression. In fact, hypoxia-responsive elements (34) are found in the promoter region of human and mouse heparanase genes, indicating a transcriptional regulation mechanism for the gene upon hypoxia stimulation. It is of interest to characterize the promoter sequence of Spalax heparanase gene in future studies when the genome of the species becomes available.

It is hypothesized that the high heparanase activity in Spalax maybe an adaptation to hypoxia with a unique mechanism in modulating activity of growth factors, consequently angiogenesis and cell proliferation. Our analysis for interaction of FGF-2 with HS samples using a filter-binding method did not show significant difference between the HS isolated from HEK293 cells cultured under normoxia and hypoxia (Supplementary Fig. 4), suggesting that hypoxia regulated heparanase activity has a distinct substrate specificity, that may be tailored for modulation of mobilized growth factors from ECM to increase soluble/free growth factors. This is, at least to some extent, demonstrated by our result that showed enhanced MAPK activity in the Spalax Hpa-HEK293 cells maintained in serum deprived DMEM (Fig. 7), suggesting a higher availability of endogenous growth factors. This finding also supports the adaptation mechanism of heparanase in Spalax.

We believe that the information reported in this study is relevant to a better understanding of the involvement of heparanase in complex biology of cancer progression, as expression of heparanase is known to be upregulated in primary and metastatic tumors (13), which often create a hypoxic environment (35).
References


Footnotes

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Figure legends

Scheme 1. **Mode of heparanase action and deamination cleavage on a HS chain.** The upper dashed arrow corresponds to potential heparanase cleavage site. The lower dashed arrow indicates the cleavage site for HNO$_2$ at pH 3.9 and the arrows indicate the cleavage sites by HNO$_2$ at pH 1.5. The circle illustrates the trisaccharide structure resulting from cleavage by heparanase and HNO$_2$ at pH 1.5. * indicates that the hexuronic acid residue can be either GlcA or IdoA.

Fig 1. **N-sulfation pattern and distribution of Spalax HS oligosaccharides.** HS purified from Spalax organs were subjected to deamination cleavage at pH 1.5 and the resulted HS-oligosaccharides were analyzed on a Bio-Gel P-10 column, as described in “Experimental Procedures”. (A) HS-oligosaccharides derived from Spalax brain. Elution positions of size-defined heparin oligosaccharides are indicated above the peaks. The arrow indicates the additional peak. (B) Proportions of each oligosaccharide from the organs of Spalax (judaei; S60) are summarized.

Fig 2. **Disaccharide analysis of HS from Spalax organs.** The disaccharides retrieved from the deamination cleavage products of HS isolated from Spalax organs were analyzed on an ion exchange column (SAX-HPLC) as described in “Experimental Procedures”. The elution pattern of the disaccharides from lung is shown in (A); the disaccharide species are indicated on top of each peak: GSM (-GlcA2S-GlcNS-); GMS (-GlcA-GlcNS6S-); IMS (-IdoA-GlcNS6S-); ISM (-IdoA2S-GlcNS-); ISMS (-IdoA2S-GlcNS6S-). Proportions of each disaccharide species from the Spalax organs (judaei; S60) are summarized in (B).

Fig 3. **Size fractionation of HS derived from HEK293 cells before and after hypoxia treatment.** The size of [${}^3$H]glucosamine labeled HS derived from mock transfected (empty circles) and Spalax
heparanase (filled circles) transfected HEK293 cells was analyzed by gel chromatography on Superose 12. (A) cell and (B) medium fractions before hypoxia treatment. (C) Medium fraction after hypoxia treatment. The elution position of heparin (14 kDa) is indicated by an arrow.

Fig 4. Distribution of HS-oligosaccharides derived from HEK293 cells maintained under normoxia or hypoxia conditions. [3H]glucosamine labeled HS released into the culture medium were cleaved with HNO\textsubscript{2} pH 1.5 and the resulting oligosaccharides were analyzed by gel chromatography on a Bio-Gel P10 column. HS-oligosaccharides from mock-HEK293 cells cultured under normoxia (A) and hypoxia (B) conditions. HS-oligosaccharides from *Spalax* heparanase-transfected cells grown under normoxia (C) and hypoxia (D) conditions. Elution positions of size-defined heparin oligosaccharides are indicated by arrows.

Fig 5. Characterization of the trisaccharide. Separation of metabolically [3H]glucosamine labeled HS-oligosaccharides derived from *Spalax* liver (A) and kidney (B) on Bio-gel P10 column. Elution positions of size-defined heparin oligosaccharides are indicated above the peaks. The trisaccharide fraction indicated by arrows was pooled and subjected to enzymatic cleavage as described under “Experimental Procedures” and analyzed on Sephadex column. (C) Analysis of the trisaccharide from liver and kidney of *Spalax judaei* (S60) before (filled circles) and after (empty circles) treatment with a mixture of \( \beta \)-glucuronidase, \( \alpha \)-iduronidase and iduronidate-2-\( O \)-sulfatase. (D) Analysis of the trisaccharide from the brain of *Spalax galili*, (S52) before (filled circles) and after (empty circles) deacetylation and cleavage with HNO\textsubscript{2} pH 3.9. (E) Analysis of the trisaccharide from *Spalax* heparanase transfected HEK293 cells before (filled circles) and after (empty circles) treatment with \( \beta \)-glucuronidase. Elution positions of di- and tetra-saccharides are indicated by arrows.

Fig 6. Quantitative RT-PCR of mRNA expression in HEK293 cells and *Spalax* kidney. Total RNA extracted from HEK293 cells with or without exposure to hypoxia (1% \( O_2 \)) and *Spalax* kidney was analyzed for expression of *VEGF*, *NDST-1* and *heparanase* by real time RT-PCR. The value of signals was standardized against that of \( \beta \)-actin. The three first bars represent the expression of *VEGF*, *heparanase* and *NDST-1* in mock-HEK293 cells cultured under hypoxia relative to the cells cultured under normoxia conditions. The two next bars represent expression of *VEGF* and *NDST-1* in *Spalax* heparanase transfected HEK293 cells relative to mock-HEK293 cells cultured under normoxia. The last bar represents expression of *NDST-1* in *Spalax* kidney relative to mouse kidney. Error bars indicate standards deviations from at least two independent experiments performed in triplicates.
Fig 7. Phosphorylation of Erk1/2 in HEK293 cells. (A) Western blot analysis of phospho-Erk1/2 and total Erk1/2 from lysates of Mock and Spalax heparanase – transfected HEK293 cells after 24 hours serum deprivation. (B) Intensity of the bands (from three samples) was quantified and the average value was presented as fold-increase of phospho-Erk1/2 in Spalax heparansase transfected cells compared to Mock transfected cells.
Figure 1.

A

B

% of total saccharides

Oligosaccharides

Elution volume (ml)
Figure 2.
Figure 3.
Figure 5.
Figure 7.

A

Mock    Spalax Hpa
pErk 44 42
Total Erk

B

Mock    Spalax Hpa
Fold increase (pErk/Erk)
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