Original Research

Complex influence of dermatan sulphate on breast cancer cells

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Abstract

Tumor transformation and progression both lead to extracellular matrix remodeling, which is also reflected in an alteration in the proportion of dermatan sulphate (DS) and chondroitin sulphate (CS) and an accumulation of the latter. In addition, a significant increase in the 6-O-sulphated disaccharide contribution to the structure of both glycosaminoglycans has been observed. It is commonly accepted that CS is more permissive for tumor growth than DS. However, the detailed role of DS in tumor progression is poorly known. We tested the effects of structurally different DSs on the behavior of cultured breast cancer cells. At a high dose (10 μg/mL), all of the DSs significantly reduced cancer cell growth, although some differences in the efficiency of action were apparent. In contrast, when used at a concentration of 1 μg/mL, the examined DSs evoked different responses ranging from the stimulation to the inhibition of cancer cell proliferation. The highest stimulatory activity was associated with fibrosis-affected fascia decorin DS, which is characterized by a particularly high content of 6-O-sulphated disaccharides. Further reduction in DS concentration to 0.5 μg/mL preserved majority of biological effects which were apparent at a dose of 1 μg/mL. The enzymatic fragmentation of the DSs, particularly by chondroitinase AC I, abolished the impact exerted by 1 μg/mL of the intact DS chains and sometimes resulted in the opposite effect. In contrast to DSs, highly sulphated C-6-S exhibited no effect on the cancer cells. Our data revealed the complexity of the effects of DSs on breast cancer cells, which include both co-receptor activity and the prevention of vascular endothelial growth factor action. In addition, the biological effect of DSs is strongly dependent not only on the glycosaminoglycan structure but also on its content in the cancer environment.

Keywords: Dermatan sulphate, breast cancer, vascular endothelial growth factor

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Introduction

The stroma of various organs is formed by connective tissue as a framework to support organ-specific cells and influence their behavior. The latter phenomenon is, however, poorly understood. In addition, the tumor transformation of organ-specific cells and tumor progression cause marked morphological and functional remodeling of the stroma. This process, which is commonly known as a ''stromal reaction,'' includes the stimulation of fibroblast growth and disturbances in the functions of these cells that lead to changes in the composition of the extracellular matrix (ECM).[1,2] The latter are also reflected in the scar-like deposition of collagen, termed ''desmoplastic reaction,'' and in the altered profile of matrix proteoglycans (PGs), which are glycoproteins modified by sulphated glycosaminoglycan (GAG) side chain(s). The tumor-dependent remodeling of the matrix PG profile results in the aberrant deposition of versican and two members of the small leucine-rich PG (SLRP) family, namely decorin and lumican. The level of chondroitin sulphate (CS)/dermatan sulphate (DS) bearing versican, which is known to form matrix complexes with hyaluronan (HA), is elevated in most cancers.[3–7] The expression of DS/CS containing decorin, in turn, appears to be associated with the amount of tumor stroma, whereas the level of keratan sulphate harbouring lumican is higher than the decorin content at least in breast cancer.[4–9] In contrast to decorin, both versican and lumican can partly originate from tumor cells.[10,11] In addition to the disturbed expression of matrix PGs, alterations in the structure of sulphated GAGs, particularly DS and CS, are also observed in solid tumor stroma. The former GAG is a copolymer composed of two types of disaccharide units: one contains N-acetyl galactosamine (GalNAc) and glucuronate (GlcA) residues, and the other has GalNAc and iduronate (IdoA) residues. In contrast, CS harbours only units of GalNAc and GlcA.

Nevertheless, in both GAGs, the majority of GalNAc residues and some hexuronate residues are modified by sulphation. The sulphation patterns of CS/DS, as well as the extent of glucuronosyl epimerization to iduronate are variable in DS chains, depending on the activities of several enzymes that undergo changes in various physiological and pathological events. The most prominent stromal GAG alteration associated with various neoplasms is a marked reduction in the DS proportion coexistent with a significant increase in the CS content.[5–7,12] In addition, the shortening of the DS/CS chains and an enhanced contribution of unsulphated and 6-O-sulphated disaccharide units to the DS/CS structure are also detected.[5–7,12–14]

It is hypothesized that stromal changes modulate cancer progression. For example, the abundant deposition of versican or the low expression of lumican and particularly decorin in breast cancer patients are associated with poor prognosis.[3,15] Moreover, a negative correlation between the decorin content and cancer progression was also found for spindle-cell sarcomas.[16] The widespread expression of decorin in tissues is considered a strong inhibitor of tumorigenesis. The transfection of various cancer cells to express decorin yields the induction of cyclin-dependent kinase inhibitor p21 and cell growth arrest.[17] The anti-tumor effects of the decorin core protein are particularly well evidenced.[18–23] Moreover, similar activity is also demonstrated by a core protein of biglycan[24–26] which is another DS-containing member of SLRP family. In contrast to their core proteins, the impact of the decorin and biglycan DS chains on tumor cell behavior is poorly understood. Similarly, the relevance of the reduction in the DS content and the structural remodeling of the GAG, both of which are observed in the tumor environment, on cancer growth is unclear. On the other hand, DS demonstrates a marked binding potential based on both high density of negative electric charge and the presence of conformationally flexible IdoA in the GAG chains. It was previously shown[27–29] that decorin DS binds hepatocyte growth factor (HGF), fibroblast growth factor-2 (FGF-2), and platelet-derived growth factor, which are also important for tumor progression.[20,30] Moreover, at least with two of these growth factors, the DS chains can function as their co-receptors.[31,32] In addition, the decorin (and biglycan) DS together with the core protein regulates collagen fibrillogenesis,[33] which can enhance the cancer-associated desmoplastic reaction leading to the inhibition or at least attenuation of cancer progression.[30] Moreover, DS can affect cell apoptosis. It was recently shown that the decorin GAG is responsible for the induction of apoptosis and/or the inhibition of proliferation in airway smooth muscle cells.[34] However, the relation between DS impact on the cell (also including cancer cell) behavior and the GAG structure is poorly understood. Thus, all of the above-mentioned circumstances prompted us to test the influence of several structurally different DSs (also derived from decorin and biglycan) which were used in several concentrations, on the growth of two widely studied human breast cancer cell lines, namely T47D and MCF-7. We chose these oestrogen-dependent cells because most primary breast tumors are sensitive to the hormone.[35]

Materials and methods

Reagents and materials

Urea, guanidine hydrochloride, Sepharose CL-4B, chondroitinase B from *Flavobacterium heparinum*, pronase, papain, standard DS from porcine intestinal mucosa, standard C-6-S from shark cartilage, Tween 20, acrylamide, azure A, phosphate buffered saline (PBS), (3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate hydrate (CHAPS), 2-aminoacridone, sodium cyanoborohydride, dimethyl sulfoxide (DMSO), ammonium acetate for HPLC, standard CS/DS disaccharides (Δdi-0S sodium salt, Δdi-4S sodium salt, Δdi-6S sodium salt, Δdi-UA-2S sodium salt), insulin, fetal bovine serum, and trypsin were purchased from Sigma-Aldrich (USA). Human recombinant vascular endothelial growth factor $(VEGF)_{165}$ was obtained from PeproTech (UK). DEAE-Sephacel, octyl-Sepharose, and Sephadex G-15 were supplied by Pharmacia Biotech (Sweden). Dimethylmethylene blue, tris(hydroxymethyl)aminomethane (Tris), standard heparin, standard C-4-S from whale cartilage, N,N′-methylenebisacrylamide, protease inhibitor cocktail containing 4-(2-aminoethyl)benzensulfonyl fluoride hydrochloride, aprotinin, bestatin, N-(trans-epoxysuccinyl)-L-leucine 4-guanidinobutylamide, leupeptin, and pepstatin A were purchased from Serva (Germany). Chondroitinase AC I from Flavobacterium heparinum was obtained from Seikagaku (Japan). Standard CS/DS disaccharides (ΔHexA(2S)-GalNAc(4,6S), ΔHexA(2S)-GalNAc(4S), ΔHexA(2S)-GalNAc(6S), ΔHexA-GalNAc(4,6S)) were obtained from Iduron (UK). Macro-Prep t-butyl hydrophobic interaction chromatography support was purchased from Bio-Rad Laboratories (USACulture media, antibiotics, and amino acids were purchased from Lonza (Belgium). Remaining chemicals were supplied by POCH (Poland).

Preparation of DSs and characterization of their structure

Samples of human fibrosis-affected palmar fascia were obtained from patients treated operatively for Dupuytren fibromatosis. The study protocol was approved by the Regional Ethical Committee. DSs from human fibrous fascia decorin and biglycan as well as DS from porcine skin biglycan were isolated as described previously.[28] In brief, small DSPGs were extracted from porcine tissue and from combined human fascia samples with buffered 7.8 M urea solution containing protease inhibitors and then fractionated firstly by an anion exchange chromatography on DEAE-Sephacel, subsequently by gel filtration on Sepharose CL-4B and finally by hydrophobic interaction chromatography on octyl Seharose CL-4B. To release GAG chains, the digestion of PG core proteins with pronase was performed in 0.05M Tris HCl buffer, pH 7.5, at 37 °C for 24 h. After thermal inactivation of the enzyme DSs were separated from protein degradation products by chromatography on DEAE-Sephacel in 0.05M TrisHCl buffer pH 7.5, containing 0.25M NaCl. Adsorbed GAGs were subsequently eluted in buffered 1.5 M NaCl, dialyzed and lyophilized. CS/DS were quantified by dimethylene

blue reaction.[36] Then, the isolated DSs and commercial DS from porcine intestinal mucosa were characterized in respect of their glucuronosyl epimerization and sulphation patterns as described previously.[29] In brief, the examined DSs were degraded through bacterial lyases chondroitinase AC I and chondroitinase B. Enzyme resistant products, representing IdoA blocks and GlcA blocks, respectively, were further resolved on gradient polyacrylamide gel electrophoresis (PAGE)[37] followed by staining with azure A/ammoniacal silver[38] and densitometric analysis. In turn, to assess the sulphation patterns of GlcA and IdoA sections, disaccharides obtained after the treatment of the examined DS with chondroitinase AC I and chondroitinase B, respectively, were labeled with fluorophore 2-aminoacridone[39] and subjected to reverse phase high performance liquid chromatography (RP HPLC) on PLRP-S 300 A column (4.6 mm × 150 mm; Polymer Laboratories, Varian, Shropshire, UK) equilibrated in 0.1 M ammonium acetate, run on a Varian ProStar HPLC system. After washing of the column with 2 mL gradient of 0–10% (v/v) methanol, disaccharides were eluted with 50 mL linear gradient of 10–30% (v/v) methanol and detected by in-line fluorescence (excitation at 425 nm, emission at 520nm).[39]

Cell cultures

T47D and MCF-7 breast cancer cells obtained from ATCC were cultured in RPMI 1640 or mixture (1:1) of RPMI 1640 and DMEM, respectively, at 37 °C, in a humidified atmosphere of 5% CO_2. Culture media were supplemented with 10% of fetal bovine serum (FBS), 0.01 mg/mL of recombinant human insulin, 1000 UI/mL of penicillin and 10 mg/mL of streptomycin, Confluent cultures were harvested by trypsinization with 0.25% EDTA-trypsin. Cells were collected by centrifugation at 800 × g for 2 min and quantified. To examine the GAG effects on a cell viability, the cells were seeded in 96-well plates (Thermo Scientific Nunc, USA) at a density of 2000 (T47D) or 5000 (MCF-7) cells per well, in 100 μL of growing medium with 10% FBS and left to grow for 24 h. Then, the culture medium was replaced by serum-free one and the cells were starved for the next 24 h. Subsequently, the medium was changed to that supplemented with various concentrations of the examined GAGs. MCF-7 cells were left to grow for 24 h in the exposure to the GAGs while T47D cells were cultured for 48 h with daily exchange of GAG containing culture medium. The viability of cancer cells was estimated by WST-1 test (Roche, Switzerland). The absorbance at 450 nm was measured after 3 h incubation of cell cultures with the test reagent. To assess the effect of IdoA and GlcA blocks on the T47D cells, in some cultures the cancer cells were left to grow for 48 h in the exposure to chondroitinase AC I or chondroitinase B resistant oligosaccharides, respectively. The oligosaccharides before of their application to cell cultures were separated from small size products of DS degradation (i.e. disaccharides and tetrasaccharides) by gel filtration on Sephadex C-15 in 0.2M NH_4HCO_3. To explore the DS effect on VEGF-mediated increase in the T47D cell viability, the cells were cultured for 24 h in the exposure to 50 ng/mL of the growth factor alone or in the combination with the individual GAG (3.3 μg/mL).

To investigate the effect of the examined DSs on the T47D cell proliferation the commercial BrdU incorporation test kit (Calbiochem, Germany) was used. In brief, cells were cultured for 24 h in the presence of the individual DS. Subsequently, the culture medium was replaced by that containing the GAG and 20 μL of BrdU solution and cells were left to grow for the next 24 h. Then, after fixation/denaturation of the cells, a mouse anti-BrdU antibody was applied at a dilution of 1:100 and cultures were incubated for 1 h at room temperature. After extensive rinsing, a goat anti-mouse IgG conjugated with peroxidase was used at a dilution of 1:1000 and the plate was incubated for 30 minutes at room temperature. Then, wells were washed and solution of fluorogenic substrate was applied. After 30 minutes, the enzymatic reaction was stopped and fluorescence of wells was measured (excitation at 325 nm, emission at 420 nm). All of the aforementioned experiments were repeated at least in triplicate.

Analysis of DS interactions with $VEGF_{165}$ by surface plasmon resonance method

To binding assay peptidoDSs prepared from porcine and human PGs by digestion with papain were used. Before the analysis these peptidoDSs were biotinylated on their peptide portion. The reaction was conducted in the presence of 10 μL of 0.01M EZ-link Sulfo NHS-LC-Biotin (Thermo Scientific, USA) as donor of active biotin, in 0.1 M PBS pH 7.2, for 2 h at room temperature. Then, modified peptidoDSs were dialyzed and lyophilized. Biotinylation efficiency was estimated by EZ biotin quantitation kit (Thermo Scientific, USA). To examine their ability to bind VEGF, the biotinylated peptidoDSs were immobilized onto a streptavidin-coated sensor chip (SAP from Xantec, Germany) in the Springle Instrument (Autolab, Netherlands) and exposed to various concentrations of the growth factor. Binding assay was performed at physiological ionic strength in PBS pH 7.4, containing 0.02% Tween-20, at 21 °C until equilibrium was reached. The dissociation phase of the VEGF-DS interaction was generated by rapid replacement of the growth factor solution with running buffer. The GAG surface was regenerated between particular measurements by washing with 2M NaCl in running buffer. Kinetics parameters of examined reactions (i.e. association and dissociation rate constants k_a and k_d, respectively) were calculated using the non-linear curve-fitting software (Kinetic evaluation version 5.4) supplied with the instrument. The equilibrium dissociation constant, K_d, was obtained from the ratio of k_d/k_a. To estimate the influence of mass transport limitation on the binding experiments, the plots of relative response values as a function of used VEGF concentration were constructed[40] for all of the examined DSs. Linear dependence between these parameters observed for all of these GAGs clearly indicated that the binding is not limited by mass transport.

Statistical analysis

The data were analyzed with the Shapiro-Wilk test to verify the assumption of normal distribution. The results were

expressed as mean values ± S.D. Between-group comparisons were based on one-way ANOVA and Tukey test accepting $p < 0.05$ as significant.

Results

Structural characteristics of the examined DSs

We tested the impact of several DSs, including the GAG chains of decorin and/or biglycan extracted from fibrosis-affected human fascia and normal porcine skin, as well as commercial DS from porcine intestinal mucosa, on breast cell behavior. Before the main experiment, we analyzed the structural characteristics of these DSs, including their molecular weight, glucuronosyl epimerization and sulphation patterns. The molecular weight of the DSs was determined by the comparison of their electrophoretic mobility during gradient PAGE with that of saccharide mass markers[29] and commercial C-4-S from whale cartilage, which shows a Mr value ranging from 25 to 50 kDa. The obtained data are presented in Table 1.

To investigate the model of their block glucuronosyl epimerization, the DSs were treated with chondroitinase AC I or chondroitinase B and then subjected to resolution of the enzyme-resistant oligosaccharides by gradient PAGE. The polymerization degree of the individual degradation products was estimated through the comparison of their electrophoretic mobility with that shown by simultaneously resolved saccharide mass markers.[29] Subsequently, the electrophoretic profiles of the DS-derived oligosaccharides were subjected to densitometric analysis. The obtained data on the relative contribution of the blocks composed of IdoA- or GlcA-containing disaccharides to the composition of the individual DS are shown in Figure 1. As can be observed, both porcine DSs are characterized by a particularly high extent of block epimerization, which results in a very high proportion of IdoA blocks in relation to GlcA blocks (Figure 1(a) versus (b)). However, the porcine skin biglycan DS contains IdoA disaccharides that are almost equally distributed into short and large size sections, whereas the DS from the porcine intestinal mucosa displays a marked content of IdoA blocks of intermediate size (Figure 1a). In contrast, both human DSs (especially human decorin GAG) show a clearly lower extent of block epimerization, with IdoA-containing disaccharides assembled mainly into short chain sections (Figure 1a). In contrast, GlcA blocks in all examined DSs have only small size (Figure 1b).

The sulphation patterns of the IdoA and GlcA regions in the porcine and human DSs were examined by RP HPLC of the fluorophore-tagged disaccharides generated after the treatment of the GAGs with chondroitinase B and chondroitinase AC I, respectively. The results, which are expressed as the relative abundance of a particular disaccharide unit, revealed that the IdoA regions accumulate prominent amounts of 4-O-sulphated disaccharides in all of the examined DSs (Figure 2a). In addition, small but clearly differential amounts of 2,4-O-disulphated disaccharides were detected, and the highest level of these units was found in the porcine biglycan DS (Figure 2a). Moreover, with the exception of the porcine biglycan DS, IdoA regions, especially those derived from human decorin, comprise detectable amounts of 4,6-O-disulphated disaccharides (Figure 2a).

In addition to IdoA regions, the GlcA segments in all of the examined DSs also contain a significant proportion of 4-O-sulphated and 2,4-O-disulphated disaccharides (Figure 2b) showing differential tissue- and PG-specific patterns (Figure 2b). Moreover, the GlcA regions in human decorin DS have a remarkable amount of 6-O-sulphated disaccharides, whereas the GlcA blocks in the porcine intestinal mucosa GAG are completely deprived of such units (Figure 2b). Additionally, in the majority of the examined DSs, the IdoA and GlcA blocks harbour small amounts of unsulphated disaccharides.

In addition to the assessment of the DS sulphation patterns, the HPLC analysis of the fluorophore-tagged disaccharides allowed the estimation of the total content of IdoA containing disaccharides, and this measure was calculated as a proportion of the amount of disaccharides generated only by chondroitinase B to the total amount of disaccharides released by both chondroitinase B and AC I. The obtained data are shown in Table 1. As can be observed, markedly lower content of IdoA units was found in the human decorin DS.

Table 1 Structural characteristics of dermatan sulphates (DSs) from human decorin and biglycan and from porcine tissue proteoglycans

DS	Range of molecular masses (kDa)	Total content of IdoA containing disaccharides (%)
Human decorin DS	7.3–28.9	78.0 ± 11
Human biglycan DS	6.9–22.5	92.1 ± 0.2
Normal porcine skin biglycan DS	7.7–37	87.7 ± 0.8
Porcine intestinal mucosa DS	25–47	92.2 ± 0.2

Note: The molecular weight of the DSs was determined by a gradient polyacrylamide gel electrophoresis as detailed under the Materials and methods section. The total content of iduronate (IdoA) residue containing disaccharides was calculated as a proportion of the amount of disaccharides generated only by chondroitinase B to the total amount of disaccharides released by both chondroitinase B and AC I as described under the Materials and methods section.

Impact of GAGs on the behavior of breast cancer cells

The effect of structurally different DSs on the viability of breast cancer cells was tested in MCF-7 and T47D cells exposed to small (0.5 μg/mL and 1 μg/mL) and high (10 μg/mL) concentrations of these GAGs. In a preliminary experiment also, higher concentrations of some GAGs including both porcine DSs and the total DS/CS, prepared from fibrosis-affected human fascia, were applied. The total fascia DS/CS is a mixture of the total decorin and biglycan GAG chains. However, due to the fact that cell responses induced by 10 μg/mL and higher concentrations of DSs (i.e. 20 and 35 μg/mL) were similar, and we had limited amounts of the decorin and biglycan DSs from fibrosis-affected human fascia, we decided to apply only the former concentration as a so called high concentration of the examined GAGs. Moreover, taking into account the

fact that tumorigenesis is most frequently associated with a prominent deposition of CS in the ECM, we also tested the effect of commercial shark cartilage C-6-S with a known sulphation pattern[28] on breast cancer cell function. This GAG is characterized by a particularly high content of 2,6-O-disulphated and 6-O-sulphated disaccharides (14% and 48% of the total disaccharides, respectively) and by a high total sulphation (117 sulphates/100 disaccharides versus 104 and 107 sulphates/100 disaccharides in the case of human and porcine DSs, respectively).[28] In addition

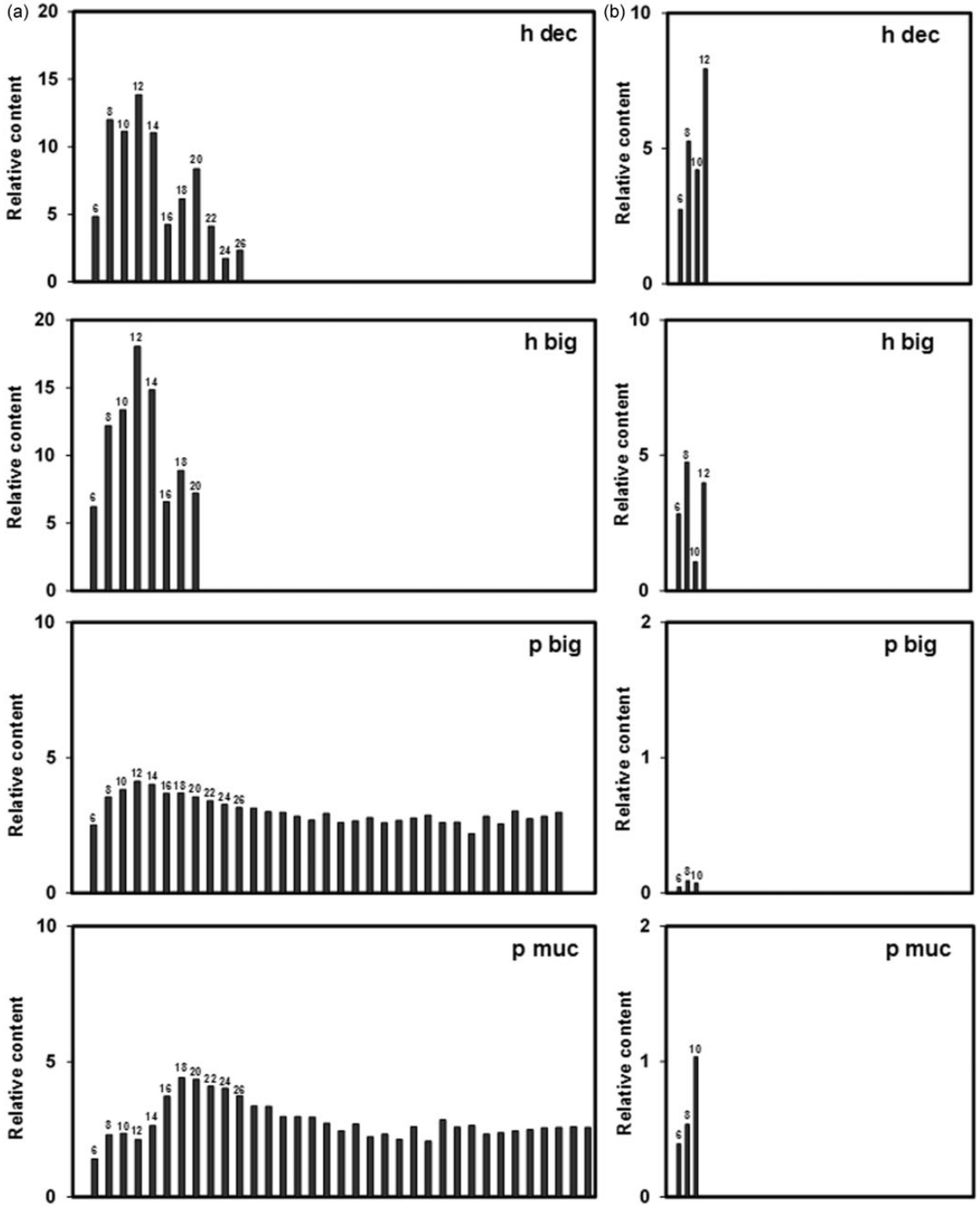

Figure 1 Comparison of block epimerization patterns characterizing human decorin dermatan sulphate (DS) (h dec), human biglycan DS (h big), porcine biglycan DS (p big), and porcine intestinal mucosa DS (p muc). DS derived oligosaccharides resistant to chondroitinase AC I (a) or chondroitinase B (b) were resolved using PAGE and subjected to densitometric analysis as described in the Materials and methods section. The data are presented as percentage contribution of particular oligo to the total content of chondroitinase AC I and B-resistant oligos. Numbers placed over some bars refer to polymerization degree of appropriate oligos. This parameter was calculated from the comparison of electrophoretic mobilities demonstrated by DS degradation products and oligosaccharide size markers, resolved in parallel.[29] Unlabeled bars show the relative proportion of longer size products of DS degradation

to C-6-S, standard heparin was also applied in some of the cell culture experiments. Because the effect of heparin on cancer cell behavior has been widely studied, this GAG can act as a reference material for the action of DSs. Before the experiment, we confirmed that cells of both lines are unable to secrete DSs as a result of the complete resistance of the culture medium components to chondroitinase B action, as monitored by the absorbance at 232 nm (data not shown).

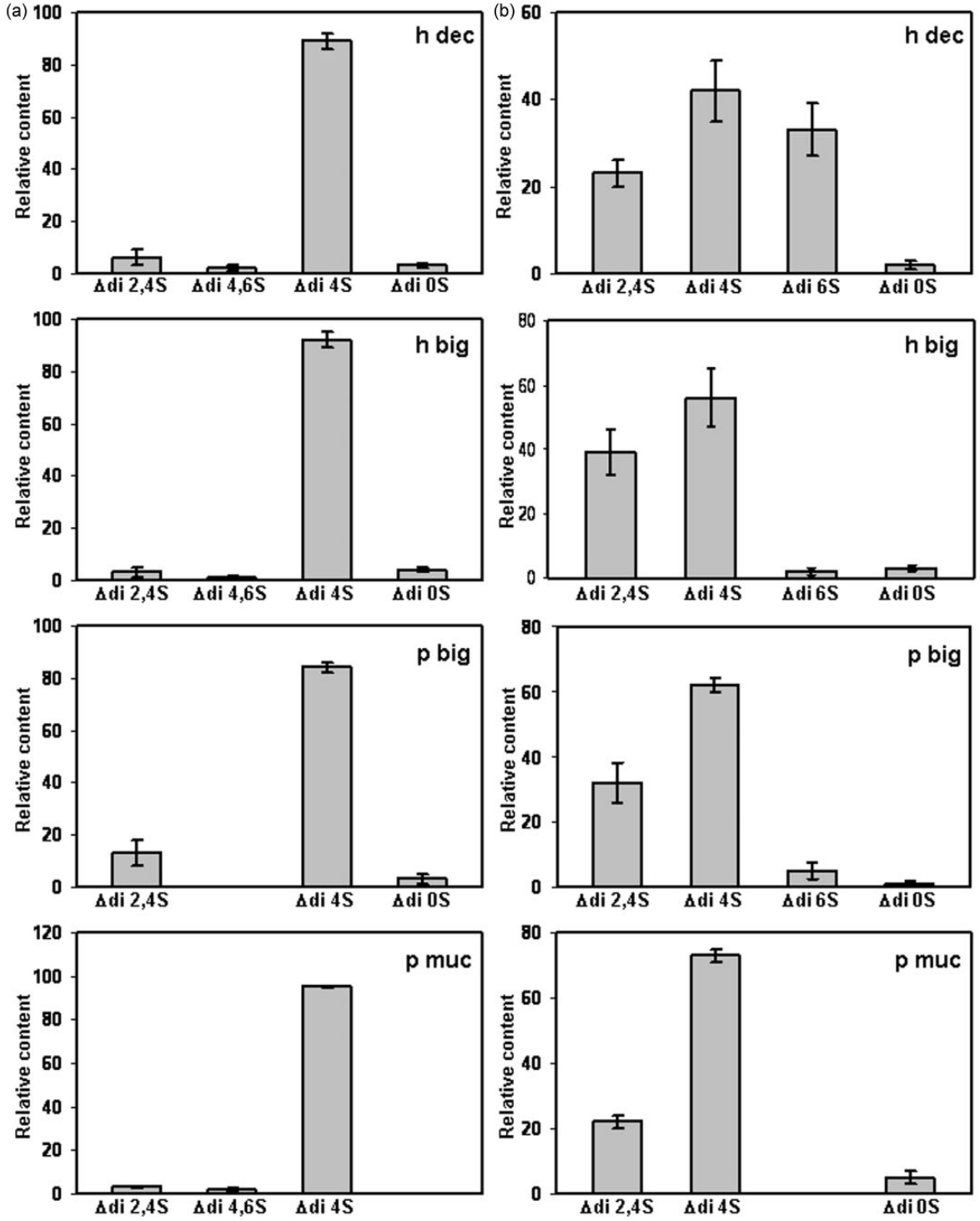

Figure 2 Comparison of sulphation patterns characterizing blocks of iduronate (a) and glucuronate (b) disaccharides in human decorin dermatan sulphate (DS) (h dec), human biglycan DS (h big), porcine biglycan DS (p big), and porcine intestinal mucosa DS (p muc). Disaccharides obtained after DS degradation through chondroitinase B (a) or chondroitinase AC I (b) were tagged with fluorophore 2-aminoacridone and fractionated using reverse phase HPLC as described in the Materials and methods section. In parallel, standard disaccharides of different sulphation type were resolved. The data are presented as percentage contribution of individual disaccharide to the total disaccharide units generated by chondroitinase B or chondroitinase AC I, respectively. Δdi2,4S – ΔHexA(2S)-GalNAc(4S); Δdi4,6S – ΔHexA-GalNAc(4,6S); Δdi4S – ΔHexA-GalNAc(4S); Δdi6S – ΔHexA-GalNAc(6S); Δdi0S – ΔHexA-GalNAc(0S) (where ΔHexA – 4-deoxy-α-L-threo-hex-4-enepyranosyluronic acid; GalNAc – N-acetyl galactosamine)

The results on the influence of DSs on the viability of the breast cancer cells MCF-7 and T47D are presented in Figures 3(a) and (b), respectively. These data are expressed as the percentage of the viability of the control cells grown in the absence of the GAGs. As shown in Figure 3, the responses of both breast cancer cell lines demonstrate DS type- and dose-dependent behavior. When applied at a high concentration (10 μg/mL), almost all of the DSs exerted a significant inhibitory effect on cancer cell viability (Figure 3). Only a high dose of porcine mucosa DS insignificantly reduced the MCF-7 cell viability (Figure 3a). However, the prolongation of the cell exposure time to 48 h made the effect of this GAG significant (data not shown). Despite the similarity in the characteristics of the cell responses generated by a high dose of all of the examined DSs, some differentiation with respect to the efficiency of the action between individual GAGs was apparent. Compared with the strong reduction in the viability of both MCF-7 and T47D cells obtained with high concentration of human decorin DS, high concentration of the porcine mucosa DS displayed the weakest effects (Figure 3). However, a particularly high level of differentiation in the biological effects was observed when the DSs were added at a concentration of 1 μg/mL to the cell cultures. As shown in Figure 3, the porcine mucosa DS at this dose manifested a mild inhibitory influence on the breast cancer cell viability, and this effect was significant only in the T47D cells. In turn, the porcine biglycan GAG exerted a neutral effect, whereas human decorin DS induced a positive response in both breast cancer cell lines (Figure 3). In contrast, human biglycan DS only significantly stimulated the viability of MCF-7 cells (Figure 3). When a concentration of the examined DSs was further reduced to 0.5 μg/mL, the majority of these GAGs showed their biological influence similar to that observed at 1 μg/mL. Only the porcine mucosa DS significantly altered its effect especially toward the T47D cells from a mild inhibitory effect apparent at a concentration of 1 μg/mL to a neutral one. Despite the similar characteristics of the alterations exerted by the individual DSs on the viability of both MCF-7 and T47D cells, the susceptibility of these cells to the GAGs was different. The prolongation of the exposure time to 48 h was required for the T47D cells to respond to the DSs at an intensity similar to that demonstrated by the MCF-7 cells grown for 24 h in the presence of the GAGs.

In contrast to the DSs, C-6-S, regardless of the concentration, did not affect the viability of any of the tested breast cancer cell lines (Figure 3). Similarly, standard heparin at a

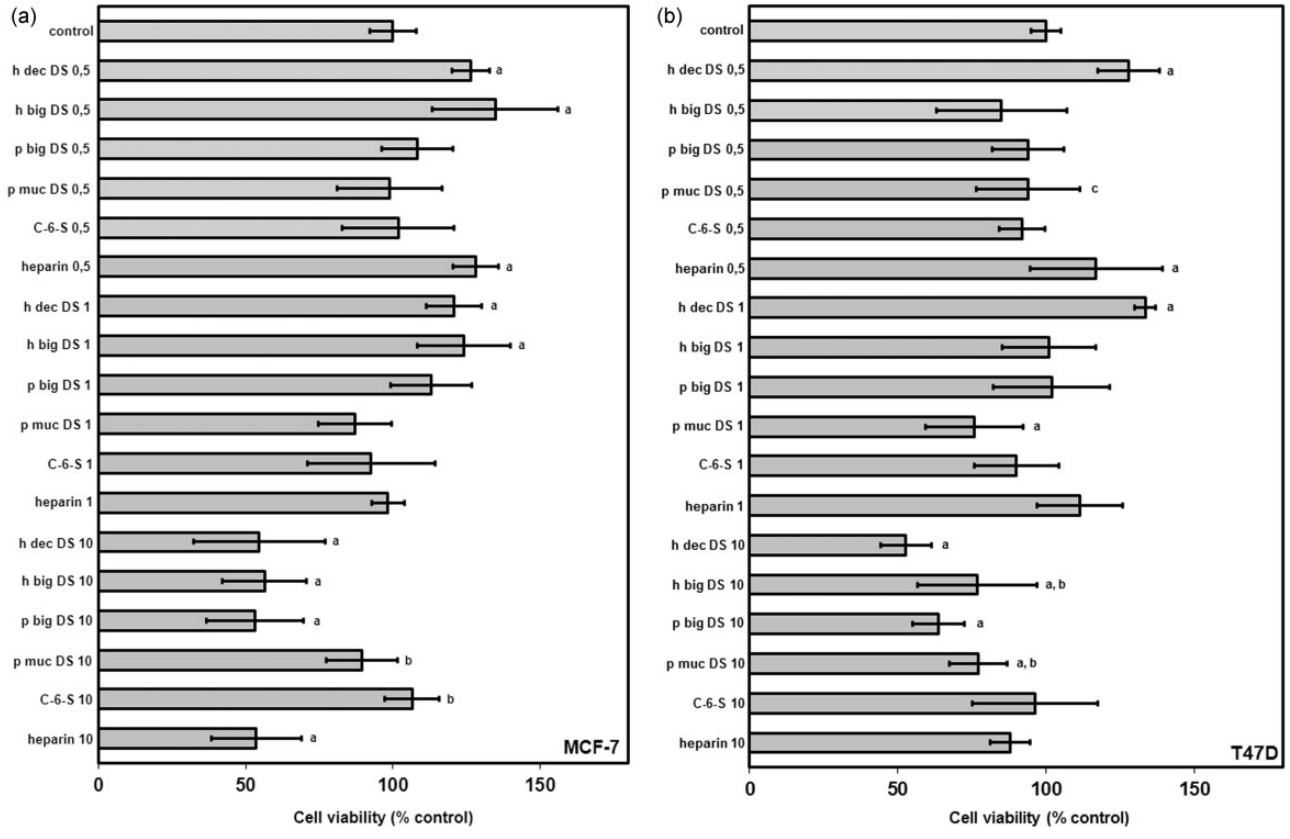

Figure 3 Effects of structurally different dermatan sulphates (DSs) on MCF-7 (a) and T47D (b) cell viability. Serum-starved cells were treated for 24 h (a) or 48 h (b) with human decorin DS (h dec DS), human biglycan DS (h big DS), porcine biglycan DS (p big DS), porcine intestinal mucosa DS (p muc DS), standard chondroitin-6-sulphate (C-6-S), and heparin. All of the glycosaminoglycans were used at three concentrations: 0.5 μg/mL, 1 μg/mL and 10 μg/mL. The cell viability was estimated using the WST-1 test as described in the Materials and methods section. The data are expressed as the means (percent of control) ± S.D. of at least three independent experiments. Statistical significance: a – compared with the control, $p < 0.05$; b – compared with 10 μg/mL human decorin DS, $p < 0.05$; c – compared with 1 μg/mL porcine intestinal mucosa DS, $p < 0.05$

concentration of 1 μg/mL failed to significantly alter the breast cancer cell viability (Figure 3). In contrast, heparin at a concentration of 0.5 μg/mL significantly stimulated both cell lines (Figure 3), whereas at a dose of 10 μg/mL it displayed significant inhibitory influence only toward MCF-7 cells (Figure 3a).

To address the mechanism(s) underlying the observed effects on breast cancer cell viability, we examined the impact of the DSs on cell proliferation, which was evaluated through the incorporation of BrdU into the DNA of the cells grown in the presence of these GAGs. However, we only tested the T47D cell proliferation due to the existence of significant similarity between these cells and the MCF-7 cells with respect to the characteristics of the responses exerted by the DSs. The obtained data (Figure 4) indicate that the influence of the individual DSs on DNA synthesis in the T47D cells exhibits good correlation with the effect evoked by the corresponding GAG on cell viability (Figure 4 versus 3(b)). Thus, the DS-mediated influence on breast cancer cell viability is propagated mainly by the modulation of the proliferation of these cells. In addition to the DS effects, the data presented in Figure 4 also show that heparin at a concentration of 10 μg/mL significantly reduced T47D cell proliferation. Some discrepancy between the data on the impact of a high dose of heparin on the proliferation and viability of T47D cells may result from the additional effect of the GAG on cell survival.[41]

Previously, Denholm et al.[42] reported that IdoA blocks in DSs are involved in the GAG-mediated stimulation of fibroblast proliferation and that their enzymatic destruction abrogates the DS effect. Moreover, the enzymatic elimination of both IdoA and especially GlcA regions from cell-associated CS/DSs also affects the proliferation of endothelial cells and some cancer cells.[43] The modulation of cell proliferation is responsible for the observed alterations in the viability of T47D cells grown in the presence of all of the examined DSs (Figure 4), which display differential patterns of glucuronosyl epimerization (Figure 1). Thus, we tested the effects exerted on breast cancer cell viability by these GAGs after they are enzymatically deprived of IdoA or GlcA blocks. Chain regions resistant to an enzyme (chondroitinase B or AC I, respectively) were then applied to the cell cultures in amounts corresponding to those present in the intact DS, which was added at a concentration of 1 μg/mL. The obtained results show (Figure 5) that the IdoA blocks released from almost all of the examined DSs evoked significant effects, which were, however, opposed to the impact exerted by their parent GAGs at the same concentration (Figure 3B). For example, the IdoA regions prepared from human decorin and biglycan promoted a reduction in the breast cancer cell viability (Figure 5), whereas the IdoA blocks from the porcine mucosa DS evoked a positive response in the cells (Figure 5). In addition to the IdoA regions, the GlcA segments from both human DSs also demonstrated an inhibitory effect on the breast cancer cells (Figure 5).

As observed in the present study, the effects of the examined DSs on T47D cell proliferation (Figure 4) may at least partially result from the known ability of DSs to regulate the activity of some growth factors. VEGF is a growth factor that is able to both induce T47D (and MCF-7) cell proliferation[44] and bind CS/DS.[45,46] Thus, we tested the impact of the examined DSs on this growth factor. First, we used the plasmon surface resonance (SPR) method to test the ability of the examined DSs to bind VEGF. In the applied system, the DS chains were immobilized onto the sensor chip and exposed under physiological ionic strength conditions to varying concentrations of VEGF. Based on the obtained sensorgrams (Figure 6), the binding reaction parameters were calculated using the monophasic 1:1 fitting model. The

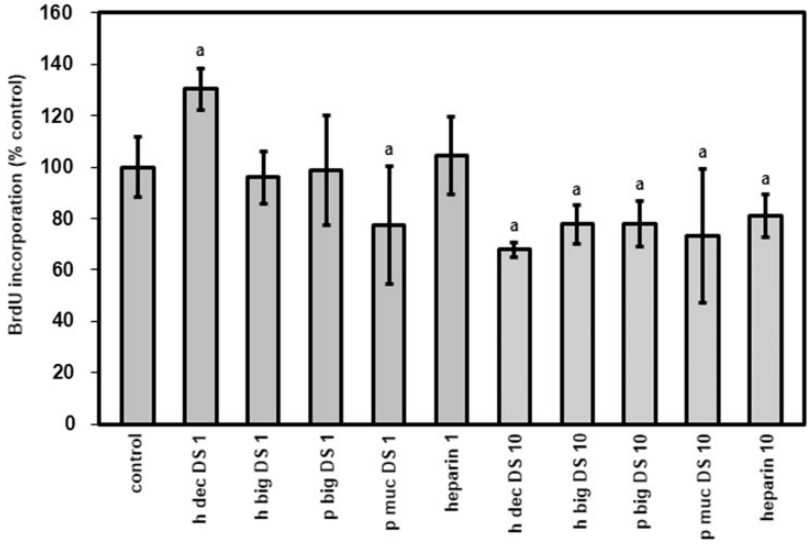

Figure 4 Effects of structurally different dermatan sulphates (DSs) on the proliferation of T47D cells. Serum-starved cells were treated for 48 h with the indicated doses (1 μg/mL and 10 μg/mL) of human decorin DS (h dec DS), human biglycan DS (h big DS), porcine biglycan DS (p big DS), porcine intestinal mucosa DS (p muc DS), and standard heparin. The cell proliferation was assessed through the BrdU incorporation assay as described in the Materials and methods section. The data are expressed as the means (percent of control) ± S.D. of at least three independent experiments. Statistical significance: a – compared with the control, $p < 0.05$

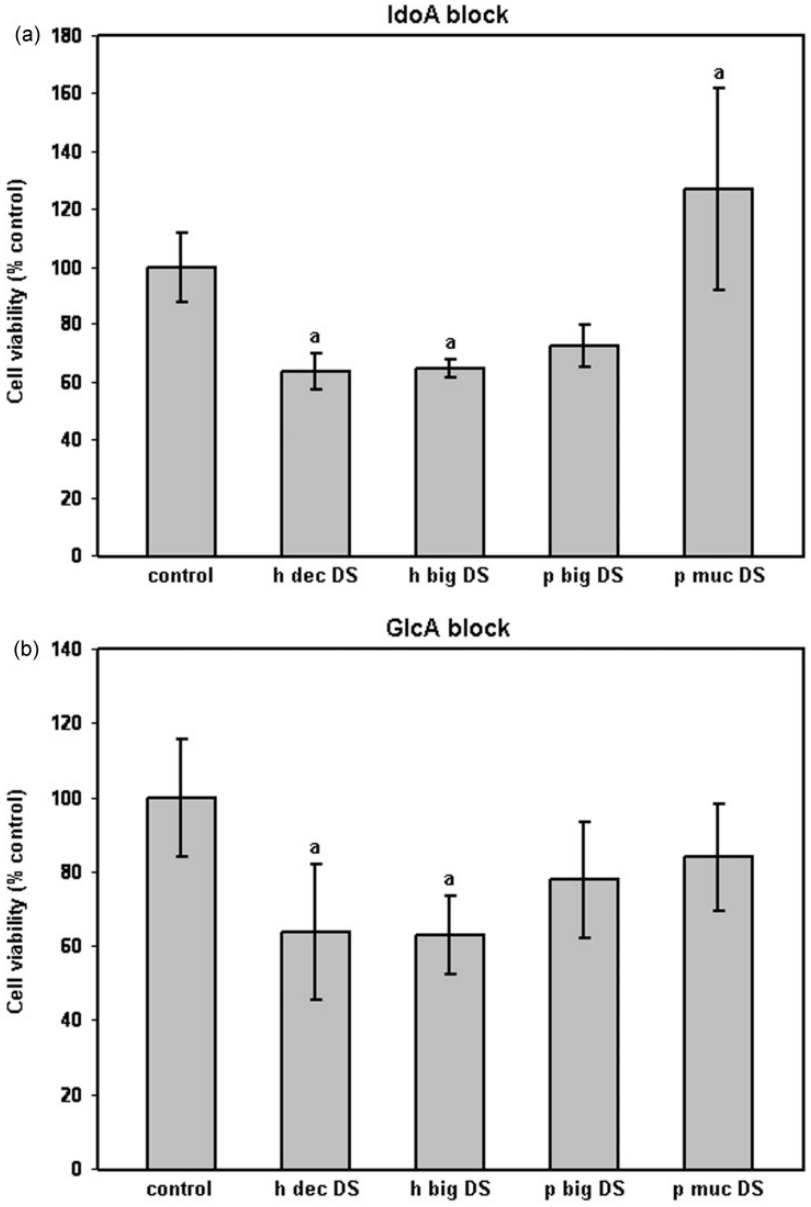

Figure 5 Effects of blocks containing iduronate (IdoA) or glucuronate (GlcA) disaccharides on T47D cell viability. Serum-starved cells were treated for 48 h with IdoA or GlcA regions prepared through the enzyme treatment of human decorin DS (h dec), human biglycan DS (h big), porcine biglycan DS (p big), and porcine intestinal mucosa DS (p muc). All of the chain sections were used at a concentration corresponding to a parent DS concentration of 1 μg/mL. The cell viability was estimated by the WST-1 test as described in the Materials and methods section. The data are expressed as the means (percent of control) ± S.D. of at least three independent experiments. Statistical significance: a – compared with the control, $p < 0.05$

obtained data (Table 2) show that the decorin DS manifests an especially high affinity to VEGF, whereas the other DSs bind the growth factor weaker (Table 2) but these interactions are as strong as that mediated by heparin.[40] Furthermore, the assessment of the DS binding capacity (Table 2) revealed that the highest number of VEGF binding sites is localized on the porcine intestinal mucosa DS, whereas the other GAGs, particularly the human biglycan DS, harbour a markedly lower number of such sites. To investigate the biological relevance of the above-mentioned binding, we tested whether the examined DSs can affect the action of exogenous VEGF toward T47D cells. However, to minimalize the adverse effects of the DSs on the cancer cells, we used in this experiment a GAG dose showing weak biological activity and we shortened the cell exposure time to 24 h. Moreover, the applied VEGF concentration was the concentration that exhibits significant but not the maximal stimulatory effect on T47D cells, as determined in a preliminary experiment. Before the combined application of VEGF and the individual DS to the cell cultures, both of the compounds were preincubated together for 15 min. The examined DSs alone exerted neutral or little inhibitory influence on the cells (Figure 7a). In contrast, VEGF when used alone showed a mild but statistically significant stimulatory impact on T47D cell viability (Figure 7a). In turn, the combined application of VEGF with each of the tested DSs led to

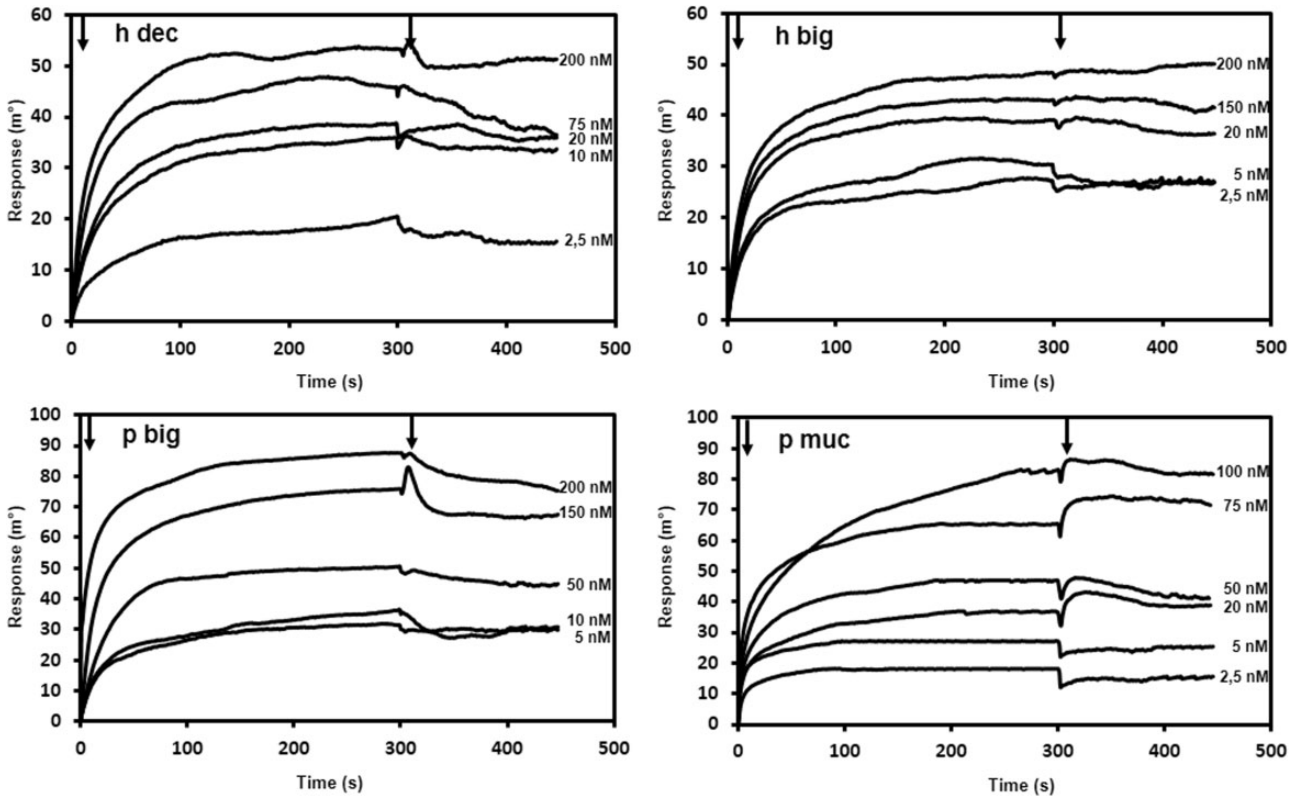

Figure 6 Plasmon surface resonance analysis of vascular endothelial growth factor $(VEGF)_{165}$ binding to dermatan sulphates (DSs). Biotinylated peptidoDSs were immobilized onto streptavidin-coated sensor chip and exposed at physiological ionic strength to varying concentrations of the growth factor as described under the Materials and methods section. h dec – sensorgrams obtained for human decorin DS; h big – sensorgrams obtained for human biglycan DS; p big – sensorgrams obtained for porcine skin biglycan DS; p muc – sensorgrams obtained for porcine intestinal mucosa DS. Arrows show the beginning of association and dissociation phases, respectively

Table 2 Kinetic parameters characterising the interactions of the vascular endothelial growth factor $VEGF_{165}$ with dermatan sulphates (DSs) from human decorin and biglycan and from porcine tissue proteoglycans

DS	Association rate constant k_a (M^{-1} s^{-1})	Dissociation rate constant k_d (s^{-1})	Equilibrium dissociation constant K_d (nM)	Binding capacity (M of VEGF/1 M of DS)
Human decorin DS	$4.1 \pm 3 \times 10^5$	$3.2 \pm 2 \times 10^{-3}$	9.3 ± 5	0.18
Human biglycan DS	$2.3 \pm 1 \times 10^5$	$7 \pm 2 \times 10^{-3}$	37.4 ± 16	0.07
Normal porcine skin biglycan DS	$5.8 \pm 2 \times 10^5$	$1.3 \pm 0.5 \times 10^{-2}$	27.6 ± 13	0.14
Porcine intestinal mucosa DS	$2.0 \pm 1 \times 10^5$	$1.0 \pm 0.9 \times 10^{-2}$	57.6 ± 21	0.6

Note: The interactions of the immobilized DSs with the growth factor were determined through the surface plasmon resonance (SPR) method, as detailed under the Materials and methods section. The interaction parameters were calculated using the nonlinear curve fitting software supplied with the SPR instrument. The molar binding capacity of a particular DS toward VEGF was determined as the ratio of the maximal molar amount of the bound VEGF (calculated by the software based on the experimental data) to the molar amount of the GAG immobilized onto the sensor chip. The amounts of interacting molecules were estimated from the following dependence: a change in the SPR response of 122 m^0 corresponds to 1 ng of mass bound to 1 mm^2 of the sensor chip. The molecular masses of particular DS species, which are needed for the calculation of the binding capacity, were evaluated through a comparison of the electrophoretic mobilities of the DSs with that of standard GAGs. The obtained average Mr values were the following: human decorin DS, 18 kDa; human biglycan DS, 15 kDa; porcine biglycan DS, 22 kDa; porcine intestinal mucosa DS, 36 kDa.

the abrogation of the growth factor-mediated effect (Figure 7a). Moreover, the same phenomenon was also observed in the cell cultures grown for 48 h in the presence of the individual DS and VEGF (Figure 7b).

Discussion

The present study provides the first comparison of the influences of structurally different DSs on breast cancer cell behavior. For the examination, we selected DSs from both normal and fibrosis-affected tissues. The fibrosis and solid-cancer-associated stromal response share some features with respect to the ECM composition, such as significant collagen and CS deposition and an increased contribution of 6-O-sulphated disaccharides to the CS/DS structure.[28,47] Thus, the DS from fibrous fascia decorin, which is characterized by a particularly high proportion

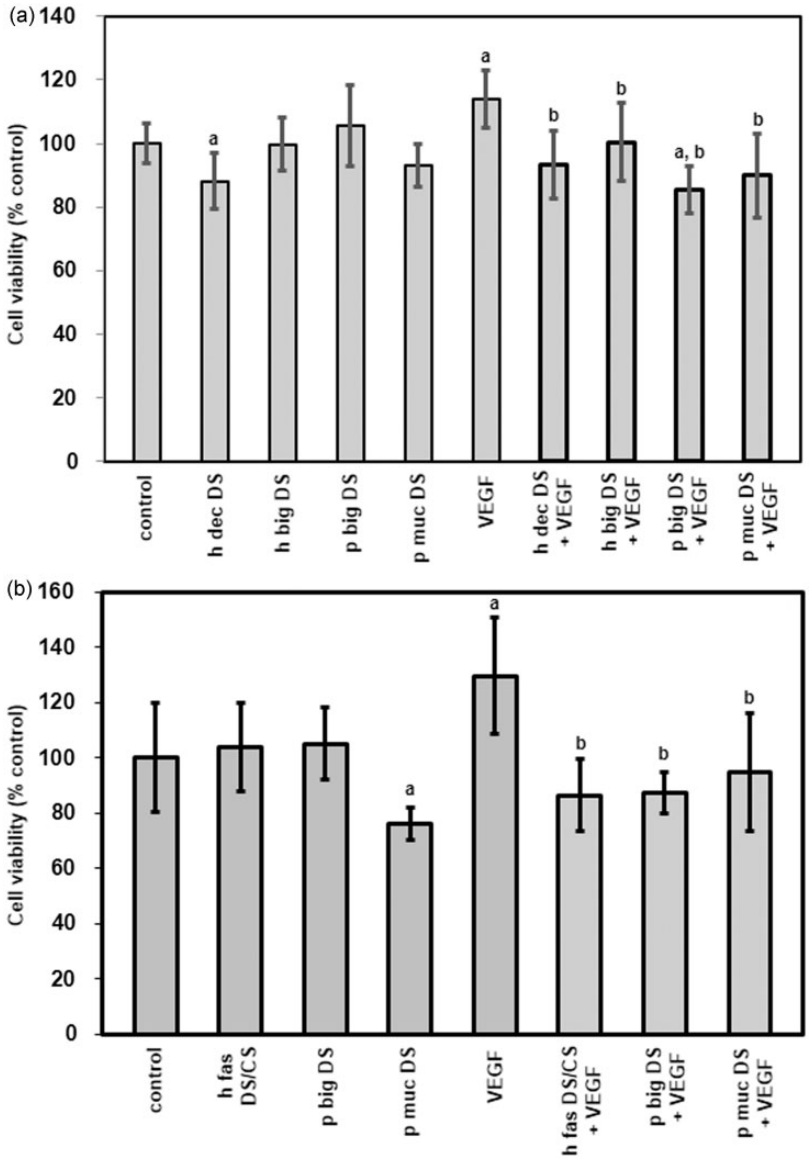

Figure 7 Effects of structurally different dermatan sulphates (DSs) on vascular endothelial growth factor $(VEGF)_{165}$-induced increase in T47D cell viability. Serum-starved cells were cultured for 24 (a) and 48 h (b) in the presence of VEGF (50 ng/mL) alone or in the combination with appropriate DS (3.3 μg/mL) as described in the Material and methods section. The data are expressed as the means (percent of control) ± S.D. of at least three independent experiments. h dec DS – human decorin DS, h big DS – human biglycan DS, h fas DS/CS – the total DS/CS from fibrosis-affected human fascia which is a mixture of decorin and biglycan GAG chains, p big DS – porcine biglycan DS, p muc DS – porcine intestinal mucosa DS. Statistical significance: a – compared to control, $p < 0.05$; b – compared to VEGF, $p < 0.05$

of 6-O-sulphated disaccharides, could to some extent mimic the remodelled structure of cancer stroma-derived GAG.[5–7,12–14] However, in contrast to the majority of solid tumors, DS and not CS is a predominant matrix GAG in fibrous tissues.[47] In addition, the expression of two SLRPs, namely decorin and biglycan, is at least retained or enhanced, respectively.[48] Moreover, tissue fibrosis is associated with a progressive reduction in cell number, and this effect may be in part induced by matrix DS.[29,47]

In the present study, we found that the function of breast cancer cells reveals some sensitivity to the structure of the DS. This phenomenon is reflected in the altered cell growth observed in response to structurally different DSs at the same concentration. Particularly high differences in the biological effects were observed between human decorin and porcine mucosa DSs. However, our results show that the mutual relationship between the structure of a DS and its final biological effect can be strongly affected by the level of the GAG in the breast cancer cell environment. A high content of almost all of the examined DSs led to a significant reduction in cell growth, independently of the GAG structure. This observation is in line with those reported by others on the influence of DS on various tumor cell lines,[49–51] which suggests that the high accumulation of the GAG in tissue can be universal inhibitor of cancer cell mitogenic activity. In contrast, both low levels of the DSs examined in this study evoked differential responses in the breast cancer cells, which were most frequently manifested as a neutral or even stimulatory effect, depending on the GAG. Interestingly, the decorin DS derived from

fibrosis-affected fascia was especially effective as promoter of breast cancer cell growth. Thus, our findings argue that the progressive reduction in DS content, which is most frequently observed in solid-tumor-associated ECM,[5–7,12] can form at least neutral if not permissive conditions for cancer growth. This is particularly important because C-6-S, which is known to accumulate in the cancer environment,[5–7,12] did not show any apparent impact on neither T47D nor MCF-7 cells.

Many studies have indicated a special role of the decorin core protein in the regulation of cancer cell behavior.[17–21] In addition, our data suggest that the decorin DS, depending on its tissue content, can exert significant and opposite biological effects on the neoplasm. However, the dual "Janus" character of the decorin DS action toward cancer cells is not unique. DS derived from fibrosis-affected fascia biglycan exerted a similar impact on MCF-7 cells. Similarly, Villena and Brandan[52] found that the DS from porcine intestinal mucosa, when used at very low concentrations, strongly increases the HGF/FGF-2-dependent proliferation of skeletal muscle satellite cells, whereas high doses of the GAG showed the opposite effect. However, the DS content in the cancer environment, which is associated with the switch in the GAG activity from the inhibition to the stimulation of cancer cell growth, may be of importance for tumor progression. Our data indicate that this tissue level of DS depends on the type of tumor, i.e. at a concentration of 0.5 and 1 μg/mL, human biglycan DS exerted a neutral effect on T47D cells but stimulated MCF-7 cells. Moreover, our data also suggest that the functional switch of decorin DS may occur at a higher level of the GAG in the tumor environment compared with the other DSs examined in the present study, particularly the DS derived from the porcine mucosa. This hypothesis is based on our finding that only decorin DS (at both 0.5 and 1 μg/mL) was able to stimulate the proliferation both MCF-7 and T47D cells. The pro-cancer action demonstrated by the human decorin DS present in a small amount could partially explain the clinical observation that a low expression of decorin in the breast cancer environment is unfavourable for a patient.[15] It is thus of interest to determine which structural features of the DS from fibrous fascia decorin could be responsible for this activity. The structural traits distinguishing the decorin DS from the other GAGs examined in this study include a high content of glucuronate residues and a particularly high level of 6-O-sulphated disaccharides. Interestingly, similar modifications are also characteristic of DSs derived from tumor-associated stroma. Unfortunately, the relevance of this type of DS remodeling, particularly with respect to the sulphation pattern, on GAG function remains obscure. Nevertheless, it is conceivable that decorin DS can be more "susceptible" to this modification than the biglycan GAG originating from the same tissue based on the structural differences between these GAGs derived from fibrosis-affected fascia.

It is generally accepted that DSs, similarly to other sulphated GAGs, can function as both regulators of growth factor bioavailability/stability and as growth factor co-receptors. Our observation that the majority of the examined DSs exerted opposite effects on breast cancer cells before and after their enzymatic fragmentation supports the co-receptor role of these GAGs. This hypothesis is also supported by the finding that high doses of all of the examined DSs lead to a reduction in breast cancer proliferation. This effect can result from the competition between individual DS chains for a simultaneous binding to ligand and its cell surface receptor. On the other hand, the reverse activity of intact DS chains and their fragments may have some practical (therapeutic) relevance. Nevertheless, further investigations are required to more fully elucidate the relationship between DS structure and function. However, it is possible that the observed weak response of T47D cells to the IdoA regions prepared from the porcine biglycan DS may result from the lack of 4,6-O-disulphated disaccharides in these fragments. The importance of 4,6-O-disulphated units in DS interactions with growth factors was reported previously.[45,46] Interestingly, our results also suggest that even a very high content of 2,4-O-disulphated disaccharides, as found in the IdoA regions of the porcine biglycan DS, can only partly compensate for the absence of 4,6-O-disulphate units.

FGF-2 is a well-known stimulator of both the T47D and MCF-7 cell proliferation.[53] In addition, we previously showed that this growth factor is a strong binding partner for DS chains from porcine and human SLRP.[29] Moreover, our unpublished data indicate that the stimulatory effects of DSs on the T47D and MCF-7 cell proliferation can be significantly attenuated in the presence of a FGFR kinase inhibitor. Thus, all of these data suggest that some effects induced by the examined DSs in the breast cancer cells can be associated with the regulation of FGF-2 loop. In addition, in several breast cancer cell lines and primary breast cancer cultures, the existence of VEGF loop and its importance for tumor cell viability were shown.[44,54] In the present study, we observed the inhibitory impact of structurally different DSs on this loop. However, further investigations must be undertaken to explain whether this DS-mediated effect directly results from the observed binding of the GAG to the growth factor. Nevertheless, our data supply direct evidence supporting a previously derived hypothesis[55] that decorin DS is a strong binding partner for $VEGF_{165}$. The ability of decorin DS to interact with the growth factor can be a manifestation of the specific cooperation between the GAG and protein portion of the PG. Previously, Khan et al.[23] showed that the decorin core protein interacts with VEGFR2 and induces downstream signaling. Thus, the binding of the natural VEGFR2 ligand (VEGF) to decorin DS could facilitate the interaction involving the decorin core protein.

In summary, our results clearly demonstrate the complex role of DS in the cancer environment and revealed some anti-cancer potential of the GAG. We hope that further investigations allow both to explain the detailed role of DS for tumor growth and to make a practical application of the GAG in cancer treatment possible.

Author contributions: EMK designed the study, conducted multiple biochemical studies, performed data interpretation and wrote the manuscript. GW carried out multiple

biochemical studies, conducted cell culture experiments and performed statistical analysis. ML carried out cell culture experiments. DK supplied tissue material. KO provided the overall advice for the study and reviewed the manuscript. All authors read and approved the final manuscript.

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REFERENCES

1. Kunz-Schughart LA, Knueschel R. Tumor-associated fibroblasts (part I): active stromal participants in tumor development and progression? *Histol Histopathol* 2002;**17**:599–621
2. Kunz-Schughart LA, Knueschel R. Tumor-associated fibroblasts (part II): functional impact on tumor tissue. *Histol Histopathol* 2002;**17**:623–37
3. Ricciardelli C, Brooks JH, Suwiwat S, Sakko AJ, Mayne K, Raymond WA, Seshadri R, LeBaron RG, Horsfall DJ. Regulation of stromal versican expression by breast cancer cells and importance to relapse-free survival in patients with node-negative primary breast cancer. *Clin Cancer Res* 2002;**8**:1054–60
4. Brown LF, Guidi AJ, Schmitt SJ, Van de Water L, Iruela-Arispe ML, Yeo TK, Tognazzi K, Dvorak HF. Vascular stroma formation in carcinoma in situ, invasive carcinoma, and metastatic carcinoma of the breast. *Clin Cancer Res* 1999;**5**:1041–56
5. Theocharis AD, Vynios DH, Papageorgakopoulou N, Skandalis SS, Theocharis DA. Altered content, composition and structure of glycosaminoglycans and proteoglycans in gastric carcinoma. *Int J Biochem Cell Biol* 2003;**35**:376–90
6. Theocharis AD. Human colon adenocarcinoma is associated with specific post-translational modifications of versican and decorin. *Biochim Biophys Acta* 2002;**1588**:165–72
7. Skandalis SS, Kletsas D, Kyriakopoulou D, Stavropoulos M, Theocharis DA. The greatly increased amounts of accumulated versican and decorin with specific post-translational modifications may be closely associated with the malignant phenotype of pancreatic cancer. *Biochim Biophys Acta* 2006;**1760**:1217–25
8. Goldoni S, Iozzo RV. Tumor microenvironment: modulation by decorin and related molecules harboring leucine-rich tandem motifs. *Int J Cancer* 2008;**123**:2473–79
9. Leygue E, Snell L, Dotzlaw H, Troup S, Hiller-Hitchcock T, Murphy LC, Roughley PJ, Watson PH. Lumican and decorin are differentially expressed in human breast carcinoma. *J Pathol* 2000;**192**:313–20
10. Ricciardelli C, Sakko AJ, Ween MP, Russell DL, Horsfall DJ. The biological role and regulation of versican levels in cancer. *Cancer Metastasis Rev* 2009;**28**:233–45
11. Nikitovic D, Berdiaki A, Zafiropoulos A, Katonis P, Tsatsakis A, Karamanaos N, Tzanakakis GN. Lumican expression is positively correlated with the differentiation and negatively with the growth of human osteosarcoma cells. *FEBS J* 2008;**275**:350–61
12. Kalathas D, Theocharis DA, Bounias D, Kyriakopoulou D, Papageorgakopoulou N, Stavropoulos MS, Vynios DH. Alterations of glycosaminoglycan disaccharide content and composition in colorectal cancer: structural and expressional studies. *Oncol Rep* 2009;**22**:369–75
13. Theocharis AD, Tsara ME, Papageorgakopoulou N, Karavias DD, Theocharis DA. Pancreatic carcinoma is characterized by elevated content of hyaluronan and chondroitin sulfate with altered disaccharide composition. *Biochim Biophys Acta* 2000;**1505**:201–06
14. Weyers A, Yang B, Yoon DS, Park JH, Zhang F, Lee KB, Linhardt RJ. A structural analysis of glycosaminoglycans from lethal and nonlethal breast cancer tissues: toward a novel class of theragnostics for personalized medicine in oncology? *OMICS* 2012;**16**:79–89
15. Troup S, Njue C, Kliewer EV, Parisien M, Roskelley C, Chakravarti S, Roughley PJ, Murphy LC, Watson PH. Reduced expression of the small leucine-rich proteoglycans, lumican, and decorin is associated with poor outcome in node-negative invasive breast cancer. *Clin Cancer Res* 2003;**9**:207–14
16. Matsumine A, Shintani K, Kusuzaki K, Matsubara T, Satonaka H, Wakabayashi T, Jino T, Uchida A. Expression of decorin, a small leucine-rich proteoglycan, as a prognostic factor in soft tissue tumors. *J Surg Oncol* 2007;**96**:411–18
17. Santra M, Mann DM, Mercer EW, Skorski T, Calabretta B, Iozzo RV. Ectopic expression of decorin protein core causes a generalized growth suppression in neoplastic cells of various histogenetic origin and requires endogenous p21, an inhibitor of cyclin-dependent kinases. *J Clin Invest* 1997;**100**:149–57
18. Csordas G, Santra M, Reed CC, Eichstetter I, McQuillan DJ, Gross D, Nugent MA, Hajnoczky G, Iozzo RV. Sustained down-regulation of the epidermal growth factor receptor by decorin. A mechanism for controlling tumor growth in vivo. *J Biol Chem* 2000;**275**:32879–87
19. Santra M, Eichstetter I, Iozzo RV. An anti-oncogenic role for decorin: down-regulation of ErbB2 leads to growth suppression and cytodifferentiation of mammary carcinoma cells. *J Biol Chem* 2000;**275**:35153–61
20. Goldoni S, Humphries A, Nyström A, Sattar S, Owens RT, McQuillan DJ, Ireton K, Iozzo RV. Decorin is a novel antagonistic ligand of the Met receptor. *J Cell Biol* 2009;**185**:743–54
21. Seidler DG, Goldoni S, Agnew C, Cardi C, Thakur MI, Owens RA, McQuillan DJ, Iozzo RV. Decorin protein core inhibits in vivo cancer growth and metabolism by hindering epidermal growth factor receptor function and triggering apoptosis via caspase-3 activation. *J Biol Chem* 2006;**281**:26408–18
22. Schönherr E, Sunderkötter C, Iozzo RV, Schaefer L. Decorin, a novel player in the insulin-like growth factor system. *J Biol Chem* 2005;**280**:15767–72
23. Khan GA, Girish GV, Lala N, Di Guglielmo GM, Lala PK. Decorin is a novel VEGFR-2-binding antagonist for the human extravillous trophoblast. *Mol Endocrinol* 2011;**25**:1431–43
24. Weber ChK, Sommer G, Michl P, Fensterer H, Weimer M, Gansauge F, Leder G, Adler G, Gress TM. Biglycan is overexpressed in pancreatic cancer and induces G1-arrest in pancreatic cancer cell lines. *Gastroenterology* 2001;**121**:657–67
25. Rectenwald ChV, Leisz S, Steven A, Mimura K, Müller A, Wulfänger J, Kiessling R, Seliger B. HER-2/neu-mediated down-regulation of biglycan associated with altered growth properties. *J Biol Chem* 2012;**287**:24320–29
26. Niedworok Ch, Röck K, Kretschmer I, Freudenberger T, Nagy N, Szarvas T, vom Dorp F, Reis H, Rübben H, Fischer JW. Inhibitory role of the small leucine-rich proteoglycan biglycan in bladder cancer. *PloS One* 2013;**8**:1–13
27. Lyon M, Deakin JA, Rahmoune H, Fernig DG, Nakamura T, Gallagher JT. Hepatocyte growth factor/scatter factor binds with high affinity to dermatan sulfate. *J Biol Chem* 1998;**273**:271–8
28. Koźma EM, Wisowski G, Olczyk K. Platelet derived growth factor BB is a ligand for dermatan sulfate chain(s) of small matrix proteoglycans from normal and fibrosis affected fascia. *Biochemie* 2009;**91**:1394–404
29. Koźma EM, Wisowski G, Kusz D, Olczyk K. The role of decorin and biglycan dermatan sulfate chain(s) in fibrosis affected fascia. *Glycobiology* 2011;**21**:1301–16
30. van Kempen LCLT, Ruiter DJ, van Muijen GNP, Coussens LM. The tumor microenvironment: a critical determinant of neoplastic evolution. *Eur J Cell Biol* 2003;**82**:539–48
31. Taylor KR, Rudisill JA, Gallo RL. Structural and sequence motifs in dermatan sulfate for promoting fibroblast growth factor-2 (FGF-2) and FGF-7 activity. *J Biol Chem* 2005;**280**:5300–06
32. Lyon M, Deakin JA, Gallagher JT. The mode of action of heparan and dermatan sulfates in the regulation of hepatocyte growth factor/scatter factor. *J Biol Chem* 2002;**277**:1040–6
33. Merline R, Schaefer RM, Schaefer L. The matricellular functions of small leucine-rich proteoglycans (SLRPs). *J Cell Commun Signal* 2009;**3**:323–35
34. D'Antoni ML, Torregiani Ch, Ferraro P, Michoud MC, Mazer B, Martin JG, Ludwig MS. Effects of decorin and biglycan on human

airway smooth muscle cell proliferation and apoptosis. *Am J Physiol Lung Cell Mol Physiol* 2008;**294**:L764–71
35. Alvarez-Baron CP, Jonsson P, Thomas Ch, Dryer SE, Williams C. The two-pore domain potassium channel KCNK5: induction by estrogen receptor α and role in proliferation of breast cancer cells. *Mol Endocrinol* 2011;**25**:1326–36
36. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim Biophys Acta* 1986;**883**:173–7
37. Laremore TN, Ly M, Zhang Z, Solakyildrim K, McCallum SA, Owens RT, Linhardt RJ. Domain structure elucidation of human decorin glycosaminoglycans. *Biochem J* 2010;**431**:199–205
38. Lyon M, Gallagher JT. A general method for the detection and mapping of submicrogram quantities of glycosaminoglycan oligosaccharides on polyacrylamide gels by sequential staining with azure A and ammoniacal silver. *Anal Biochem* 1990;**185**:63–70
39. Deakin JA, Lyon M. A simplified and sensitive fluorescent method for disaccharide analysis of both heparin sulfate and chondroitin/dermatan sulfates from biological samples. *Glycobiology* 2008;**18**:483–91
40. Zhao W, McCallum SA, Xiao Z, Zhang F, Linhardt RJ. Binding affinities of vascular endothelial growth factor (VEGF) for heparin-derived oligosaccharides. *Biosci Rep* 2012;**32**:71–81
41. Ishikawa Y, Kitamura M. Inhibition of glomerular cell apoptosis by heparin. *Kidney Int* 1999;**56**:954–63
42. Denholm EM, Cauchon E, Poulin C, Silver PJ. Inhibition of human dermal fibroblast proliferation by removal of dermatan sulfate. *Eur J Pharmacol* 2000;**400**:145–53
43. Denholm EM, Lin YQ, Silver PJ. Anti-tumor activities of chondroitinase AC and chondroitinase B: inhibition of angiogenesis, proliferation and invasion. *Eur J Pharmacol* 2001;**416**:213–21
44. Miralem T, Steinberg R, Price D, Avraham H. VEGF $_{165}$ requires extracellular matrix components to induce mitogenic effects and migratory response in breast cancer cells. *Oncogene* 2001;**20**:5511–24
45. Deepa SS, Umehara Y, Higashiyama S, Itoh N, Sugahara K. Specific molecular interactions of oversulfated chondroitin sulfate E with various heparin-binding growth factors. Implications as a physiological binding partner in the brain and other tissues. *J Biol Chem* 2002;**277**:43707–16
46. ten Dam GB, van de Westerlo EMA, Purushothaman A, Stan RV, Bulten J, Sweep FCGJ, Massuger LF, Sugahara K, van Kuppevelt TH. Antibody GD3g7 selected against embryonic glycosaminoglycans defines chondroitin sulfate-E domains highly up-regulated in ovarian cancer and involved in vascular endothelial growth factor binding. *Am J Pathol* 2007;**171**:1324–33
47. Shih B, Bayat A. Scientific understanding and clinical management of Dupuytren disease. *Nat Rev Rheumatol* 2010;**6**:715–26
48. Koźma EM, Olczyk K, Wisowski G, Głowacki A, Bobiński R. Alterations in the extracellular matrix proteoglycan profile in Dupuytren's contracture affect the palmar fascia. *J Biochem* 2005;**137**:468–476
49. Nikitovic D, Assouti M, Sifaki M, Katonis P, Krasagakis K, Karamanos NK, Tzanakakis GN. Chondroitin sulfate and heparan sulfate-containing proteoglycans are both partners and targets of basic fibroblast growth factor-mediated proliferation in human metastatic melanoma cell lines. *Int J Biochem Cell Biol* 2008;**40**:72–83
50. Nikitovic D, Zafiropoulos A, Tzanakakis GN, Karamanos NK, Tsatsakis AM. Effects of glycosaminoglycans on cell proliferation of normal osteoblasts and human osteosarcoma cells depend on their type and fine chemical composition. *Anticancer Res* 2005;**25**:2851–6
51. Fthenou E, Zafiropoulos A, Tsatsakis A, Stathopoulos A, Karamanos NK, Tzanakakis GN. Chondroitin sulfate A chains enhance platelet derived growth factor-mediated signaling in fibrosarcoma cells. *Int J Biochem Cell Biol* 2006;**38**:2141–50
52. Villena J, Brandan E. Dermatan sulfate exerts an enhanced growth factor response on skeletal muscle satellite cell proliferation and migration. *J Cell Physiol* 2004;**198**:169–78
53. Karey KP, Sirbasku DA. Differential responsiveness of human breast cancer cell lines MCF-7 and T47D to growth factors and 17β-estradiol. *Cancer Res* 1988;**48**:4083–92
54. Weigand M, Hantel P, Kreienberg R, Waltenberger J. Autocrine vascular endothelial growth factor signaling in breast cancer. Evidence from cell lines and primary breast cancer cultures in vitro. *Angiogenesis* 2005;**8**:197–204
55. Le Jan S, Hayashi M, Kasza Z, Eriksson I, Bishop JR, Weibrecht I, Heldin J, Holmborn K, Jakobsson L, Söderberg O, Spillmann D, Esko JD, Claesson-Welsh L, Kjellen L, Kreuger J. Functional overlap between chondroitin and heparan sulfate proteoglycans during VEGF-induced sprouting angiogenesis. *Arterioscler Thromb Vasc Biol* 2012;**32**:1255–63

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