Glycobiology vol. 21 no. 10 pp. 1301–1316, 2011
doi:10.1093/glycob/cwr065
Advance Access publication on May 4, 2011

The role of decorin and biglycan dermatan sulfate chain(s) in fibrosis-affected fascia

Ewa M Koźma[1,2], Grzegorz Wisowski[2], Damian Kusz[3], and Krystyna Olczyk[2]

[2]Department of Clinical Chemistry and Laboratory Diagnostics, Medical University of Silesia, ul. Jedności 8, Sosnowiec 41-200, Poland and [3]Department of Orthopedics and Traumatology, Medical University of Silesia, Katowice 40-635, Poland

Received on December 6, 2010; revised on April 19, 2011; accepted on April 27, 2011

Organ fibrosis is associated with excessive deposition of dermatan sulfate (DS) in the extracellular matrix (ECM) of the affected tissue. However, the significance of DS in fibrosis process is poorly known. Thus, we have analyzed both in vitro and in vivo the binding potential toward fibroblast growth factor-2, platelet-derived growth factor BB and fibronectin (FN) of DS representing glycosaminoglycan (GAG) chains of two proteoglycans decorin and biglycan derived from fascia undergoing fibrosis due to Dupuytren's disease. Moreover, to investigate the relation between DS structure and its binding properties to above ligands, we have also studied the interactions of the GAG chains from normal porcine skin decorin and biglycan. The examined interactions, especially those engaging extractable pool of both human and porcine decorin DS, are characterized by very high affinity and low capacity. Moreover, the presence of iduronate residues is not essential for the DS binding to all studied ligands and the interactions more strongly depend on the GAG sulfation pattern. All investigated interactions have biological relevance as judged from the coexistence of decorin (and biglycan) DS, both growth factors and FN in supra-molecular complexes localized in ECM of both fibrous and normal human fascia. Moreover, these complexes also include collagen type III. It seems that fascia fibrosis process when compared with physiological circumstances is associated with the preservation of at least some functions of decorin and biglycan DSs such as the regulation of growth factor bioavailability and most probably influence FN fibrillogenesis as well as coupling of various fibrilar matrix element assembly.

Keywords: dermatan sulfate / fibronectin / fibrosis / growth factors

Introduction

Dermatan sulfate (DS) is a glycosaminoglycan (GAG) widespread in many animal tissues mainly as a component of two extracellular matrix (ECM) proteoglycans (PGs) decorin and biglycan. The GAG consists of *N*-acetylgalactosamine (GalNAc) residues that are alternated with glucuronic acid (GlcA) or iduronic acid (IdoA) ones present in various mutual proportions (for review, see Prydz and Dalen 2000). Since great part of DS monosaccharide residues undergo sulfation, the GAG chains display the marked density of a negative electric charge. This structural feature together with differential and tissue-specific extents of DS sulfation and glucuronosyl epimerization (i.e. isomerization of GlcA to IdoA; Cheng et al. 1994) seems to determine the GAG-binding potential associated with the fulfillment of biological functions. The latter ones include both contribution to the ECM assembly and the impact on cell activity. DS as a component of decorin and biglycan participates in collagen fibrillogenesis (Danielson et al. 1997; Ameye et al. 2002) and is an element of elastic fibers (Reinboth et al. 2002). Moreover, DS chains of both above small PGs in cooperation with their core proteins regulate cell adhesion and migration (Bidanset et al. 1992; Merle et al. 1999). Some DS chains are also able to bind and/or promote the activity of growth factors such as fibroblast growth factors 2 and 7 (FGF-2 and FGF-7), hepatocyte growth factor/scatter factor and platelet-derived growth factor BB (PDGF-BB; Lyon et al. 1998; Taylor et al. 2005; Koźma et al. 2009). In addition, the recent studies have described the neuritogenic activity of various hybrid chondroitin sulfates (CSs)/DSs (Deepa et al. 2002; Hikino et al. 2003; Bao et al. 2004; Nandini et al. 2004, 2005; Ida et al. 2006). However, both the DS role in various physiological and pathological events and relation between the GAG structure and its binding properties are still poorly known.

The organ fibrosis is a complex multi-step process associated with a marked reduction in the tissue cellularity and excessive deposition of ECM components, including collagen, fibronectin (FN) and DS (Koshiishi et al. 2002; Laurent et al. 2007). Thus, such a remodeling also characterizes palmar fascia affected with Dupuytren's contracture (DC), i.e. a fibrosis connected disorder most probably of hereditary origin (for review, see Badalamente and Hurst 1999; Tomasek et al. 1999; Rayan 2007). The disease manifests by two types of lesions commonly coexisting in the affected tissue: nodules formed by intensively proliferating (myo)fibroblasts as well as

[1]To whom correspondence should be addressed: Tel: +48-32-364-11-55; Fax: +48-32-364-11-57; e-mail: mkozma@sum.edu.pl

strongly stretched thick collagenous cords localized in hypocellular areas (Rayan 2007). The former structures most probably correspond with the early DC stage, whereas the latter lesions leading to digit contracture are associated with advanced disease (Rayan 2007). The cell activity on the initial DC phases depends on the high expression of some cytokines and growth factors (Baird et al. 1993) also including FGF-2 and PDGF-BB (Gonzalez et al. 1992; Terek et al. 1995). Both growth factors are implicated in the stimulation of cell proliferation, migration and survival (for review, see Heldin and Westermark 1999; Sorensen et al. 2006). In addition, PDGF-BB also promotes the collagen synthesis (Heldin and Westermark 1999). In turn, collagen fibrillogenesis, which in the case of fibrous fascia leads to the formation of tight pseudotendinous cords, most probably depends on the FN fibril assembly (Sottile and Hocking 2002). Despite the fact that a general scheme of DC progress is clear, the detailed mechanisms underlying disease evolution are poorly known. It prompted us to investigate the role in fibrosis-affected fascia of DS representing side chains of decorin and biglycan which are the major PGs in the tissue ECM (Koźma et al. 2005). To attain this goal, we have tested the DS ability to bind FGF-2 and FN both in vitro at the physiological ionic strength as well as in vivo by an examination of composition of supramolecular complexes derived from the fibrous fascia ECM. Moreover, to the present investigation, we have included again PDGF-BB due to the fact that previously when we have examined the DS interaction with the growth factor we were unable to estimate all kinetic parameters characterizing the binding (Koźma et al. 2009). Thus, now we have completed these data.

Results

Assessment of DC-derived DS ability to bind FGF-2, FN and PDGF-BB

Small DSPGs—decorin and biglycan—extracted from the ECM of fibrosis-affected palmar fascia and purified by chromatography series were source of DS chains that interaction potential at physiological ionic strength was investigated. Binding capabilities of DS chains immobilized onto a sensor chip and exposed to varying concentration of ligands were estimated by the surface plasmon resonance (SPR) method. The sensorgrams obtained are shown in Figures 1–3, while binding reaction parameters calculated on the sensorgram basis using monophasic 1:1 model fitting are presented in Table I. As can be seen, DSs from the urea extractable portion of both DC-derived small PGs display high affinity for all investigated ligands. However, both growth factors and FN were particularly strongly bound by decorin DS. All its interactions were characterized by high association and/or low dissociation rates that yield in low and relatively similar

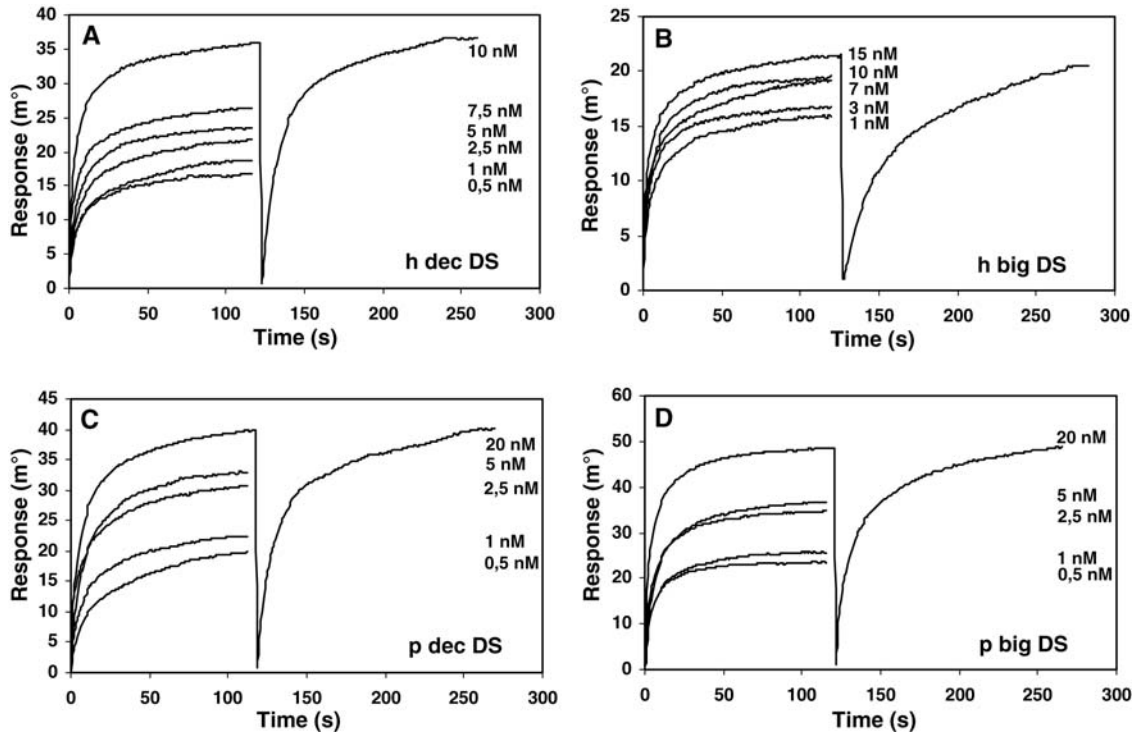

Fig. 1. Plasmon surface resonance analysis of FGF-2 binding to DS chains from human fibrous fascia and normal porcine skin small PGs. Biotinylated peptido-DSs were immobilized onto a streptavidin-coated sensor chip and exposed to varying concentrations of the growth factor at physiological ionic strength as described in *Materials and methods*. (**A**) Sensorgrams obtained for fibrosis-affected human fascia decorin DS (h dec DS) interaction. (**B**) Sensorgrams obtained for fibrosis-affected human fascia biglycan DS (h big DS) interaction. (**C**) Sensorgrams obtained for normal porcine skin decorin DS (p dec DS) interaction. (**D**) Sensorgrams obtained for normal porcine skin biglycan DS (p big DS) interaction.

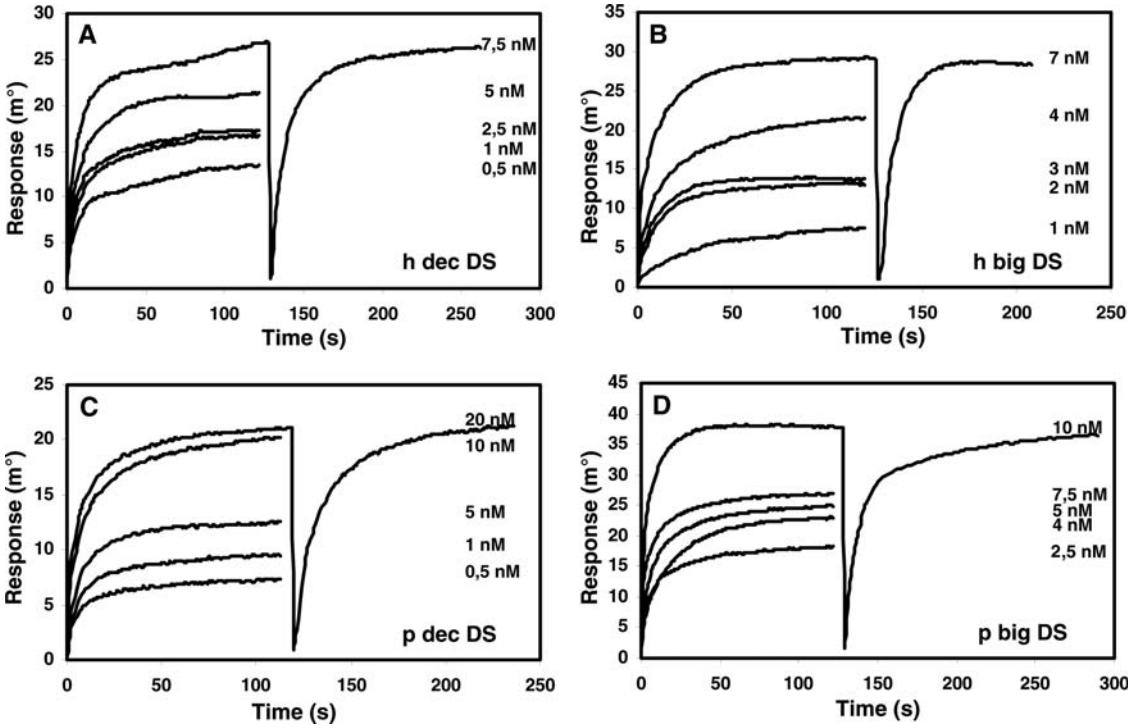

Fig. 2. Plasmon surface resonance analysis of FN binding to DS chains from human fibrous fascia and normal porcine skin small PGs. Biotinylated peptido-DSs were immobilized onto a streptavidin-coated sensor chip and exposed to varying concentrations of FN at physiological ionic strength as described in *Materials and methods*. (**A**) Sensorgrams obtained for fibrosis-affected human fascia decorin DS (h dec DS) interaction. (**B**) Sensorgrams obtained for fibrosis-affected human fascia biglycan DS (h big DS) interaction. (**C**) Sensorgrams obtained for normal porcine skin decorin DS (p dec DS) interaction. (**D**) Sensorgrams obtained for normal porcine skin biglycan DS (p big DS) interaction.

equilibrium dissociation constants (Table I). Whereas biglycan DS demonstrated especially high affinity for FGF-2 (Table I). In contrast to high affinity, all examined interactions mediated by an extractable pool of DC DSs were distinguished by low binding capacity (Table I). The phenomenon was particularly prominent in the case of decorin DS (Table I). Moreover, it is worthy of noting that significant differentiation appeared with regard to the number of binding sites for particular ligands on each DS (Table I). This observation suggests the existence of distinct requirements as to DS structure involving in the interactions.

Evaluation of DS structural properties responsible for interactions with FGF-2, FN and PDGF-BB

High affinity of DC-derived DSs to all examined ligands concomitant with differential binding capacity prompted us to investigate structural features of the GAG implicated in these interactions. Moreover, to this examination, the DS chains from normal porcine skin decorin and biglycan were also included. These DSs likewise DC GAGs strongly bound both growth factors and FN (Figures 1–3 and Table I). Furthermore, significant similarity with regard to DS-binding preferences between human and porcine PGs was apparent. Both decorins bound avidly all examined ligands, whereas biglycans especially strongly interacted with FGF-2 (Table I). In contrast to their binding properties, human and porcine DSs, however, displayed marked differences in their chain structure. Structural analysis included an evaluation of glucuronosyl epimerization and sulfation patterns. The former structural feature was assessed on the basis of free DS chain sensitivity to chondroitinase AC I or B action followed by the resolution of generated chain fragments by gradient polyacrylamide gel electrophoresis (PAGE). The obtained electrophoretic profiles are presented in Figure 4. Analysis of them revealed that both porcine DSs are characterized by very high extent of glucuronosyl epimerization resulting in high IdoA content. Such a conclusion can be drawn from the presence of marked amounts of DS chain fragments resistant to chondroitinase AC I treatment (Figure 4, lanes 8 and 9). In addition, both porcine DS species displayed remarkable sensitivity to chondroitinase B. The enzyme action yielded only a negligible number of resistant chain regions (Figure 4, lanes 2 and 3). Simultaneously, both porcine DSs were completely sensitive to the combined action of chondroitinases AC I and B (data not shown). This finding confirmed that chondroitinase B generated oligosaccharides visible on electrophoregrams (Figure 4, lanes 2 and 3) represent GlcA segments, whereas chondroitinase AC I-resistant chain fragments (Figure 4, lanes 8 and 9) are IdoA blocks. Further analysis of electrophoretic profiles obtained for both porcine skin DS species revealed approximate amounts of chondroitinase AC I-resistant chain fragments showing fast and slow electrophoretic mobility (Figure 4, lanes 8 and 9). This phenomenon more prominent in the case of porcine biglycan (Figure 4, lane 9) suggests similar contribution of short and long IdoA blocks to porcine

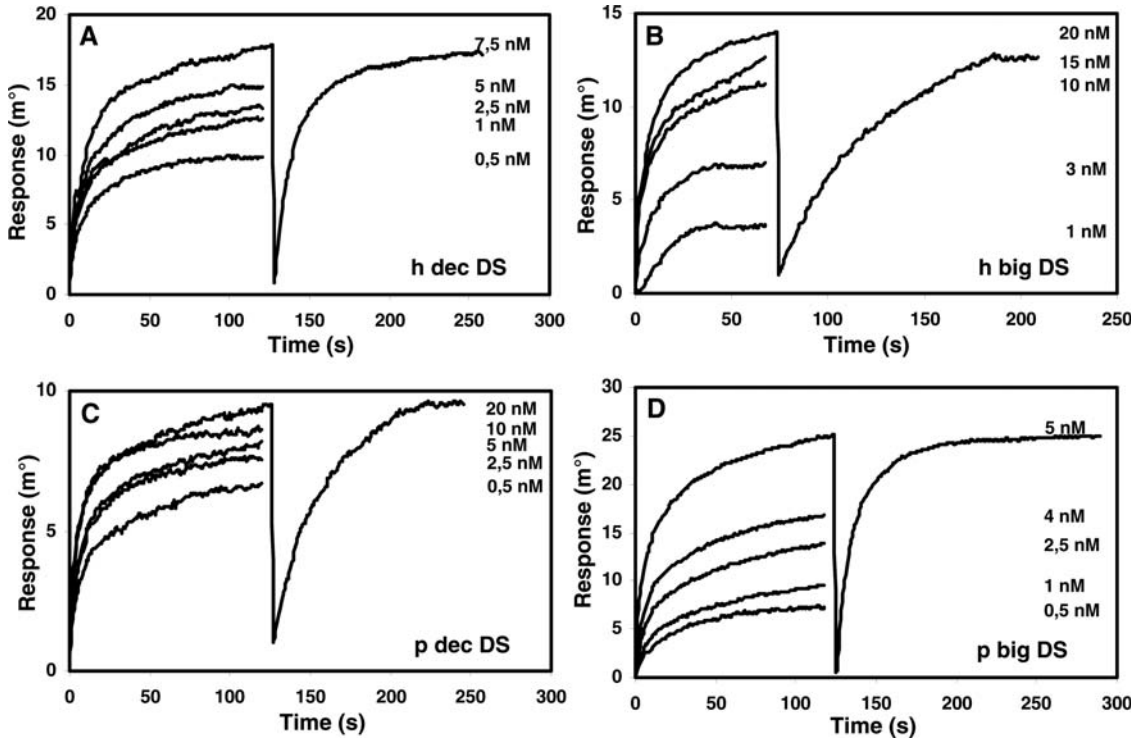

Fig. 3. Plasmon surface resonance analysis of PDGF-BB binding to DS chains from human fibrous fascia and normal porcine skin small PGs. Biotinylated peptido-DSs were immobilized onto a streptavidin-coated sensor chip and exposed to varying concentrations of the growth factor at physiological ionic strength as described in *Materials and methods*. (**A**) Sensorgrams obtained for fibrosis-affected human fascia decorin DS (h dec DS) interaction. (**B**) Sensorgrams obtained for fibrosis-affected human fascia biglycan DS (h big DS) interaction. (**C**) Sensorgrams obtained for normal porcine skin decorin DS (p dec DS) interaction. (**D**) Sensorgrams obtained for normal porcine skin biglycan DS (p big DS) interaction.

DS composition. Likewise, as porcine DSs, both DC-derived GAG species displayed complete sensitivity to the combined action of chondroitinases AC I and B (data not shown). However, in contrast to porcine GAGs human DS composition especially that of decorin DS revealed higher participation of GlcA disaccharides. Such a conclusion results from an enhanced proportion of oligosaccharides generated from human DS species by chondroitinase B (Figure 4, lanes 4 and 5) in relation to oligosaccharides released from these GAGs by chondroitinase AC I (Figure 4, lanes 6 and 7). Moreover, the majority of IdoA and GlcA disaccharides in these GAGs are assembled into rather short blocks as judged from a predominance of fast migrating oligosaccharides among the products both of chondroitinase AC I and B action (Figure 4, lanes 4–7).

Besides epimerization pattern, the sulfation one also differs porcine skin and human DC fascia DS species. The latter structural feature was examined by a reverse-phase high-performance liquid chromatography (RP HPLC) of fluorophore 2-aminoacridone (AMAC) tagged disaccharides that were products of complete DS degradation by chondroitinase ABC. The results obtained indicate that both DC decorin and DC biglycan DSs display increased contents of 4,6-*O*-disulfated, 6-*O*-sulfated and unsulfated disaccharides when compared with both porcine GAGs (Figure 5, Table II). Moreover, we found the lack of 2,6-*O*-disulfated disaccharides in porcine decorin DS and negligible content of them in porcine biglycan GAG (Figure 5, Table II). In contrast, DC-derived DSs revealed higher contribution (~0.2%) of 2,6-*O*-disulfated disaccharides (Figure 5, Table II). On the other hand, all examined DSs share some traits of sulfation patterns including similar average sulfation degree, the presence of 4-*O*-sulfated disaccharides as the predominant structural units and relatively approximated content of 2,4-*O*-disulfated disaccharides (Figure 5, Table II).

High affinity to FGF-2, FN and PDGF-BB manifested by both porcine DSs, which contain almost exclusively disaccharides with IdoA, suggests the importance of the epimer for the interactions. To verify this hypothesis, we assessed the ability of IdoA and GlcA sections of DS chains to compete with the intact GAG binding. To the examination, we have chosen DC decorin DS due to remarkable amount of both IdoA and in particular GlcA disaccharides as judged from the analysis of electrophoretic profiles generated after the GAG degradation by chondroitinases B and AC I (Figure 4, lanes 4 and 7). This suggestion was also supported by quantification of GlcA disaccharides. Their contribution to examined DS composition was calculated as the ratio of absorbance at 232 nm generated by chondroitinase AC I action on the GAG to absorbance at 232 nm obtained after the DS digestion by chondroitinase ABC. DC decorin DS contains ~17% of GlcA

Table I. Kinetic parameters characterizing the interactions of various ligands with DSs from human Dupuytren's fascia and porcine skin small PGs

Ligand	DS	Association rate constant k_a (M^{-1} s^{-1})	Dissociation rate constant k_d (s^{-1})	Equilibrium dissociation constant K_d (nM)	Number of binding sites on 1 nM of DS
FGF-2	Dupuytren's fascia decorin DS	$8.63 \pm 0.26 \times 10^6$	$1.11 \pm 0.09 \times 10^{-2}$	0.55 ± 0.11	49×10^4
	Dupuytren's fascia biglycan DS	$1.3 \pm 0.1 \times 10^7$	$3.3 \pm 1.4 \times 10^{-3}$	0.33 ± 0.1	84×10^4
	Normal porcine skin decorin DS	$5.63 \pm 0.11 \times 10^6$	$4.3 \pm 0.4 \times 10^{-3}$	0.6 ± 0.06	41×10^4
	Normal porcine skin biglycan DS	$1.1 \pm 0.02 \times 10^7$	$4.3 \pm 0.7 \times 10^{-3}$	0.7 ± 0.06	67×10^4
FN	Dupuytren's fascia decorin DS	$2.1 \pm 0.20 \times 10^6$	$1.4 \pm 0.8 \times 10^{-3}$	0.5 ± 0.1	78×10^4
	Dupuytren's fascia biglycan DS	$7.9 \pm 0.3 \times 10^6$	$9.5 \pm 0.0.4 \times 10^{-2}$	10.04 ± 2.6	108×10^4
	Normal porcine skin decorin DS	$7.66 \pm 0.35 \times 10^6$	$0.91 \pm 0.16 \times 10^{-2}$	1.3 ± 0.24	25×10^4
	Normal porcine skin biglycan DS	$2.1 \pm 0.06 \times 10^7$	$1.8 \pm 0.13 \times 10^{-2}$	2.2 ± 0.29	196×10^4
PDGF-BB	Dupuytren's fascia decorin DS	$1.24 \pm 0.1 \times 10^7$	$1.5 \pm 1 \times 10^{-3}$	0.37 ± 0.1	20×10^4
	Dupuytren's fascia biglycan DS	$3.6 \pm 0.2 \times 10^6$	$2.3 \pm 0.2 \times 10^{-2}$	4.8 ± 1.5	31×10^4
	Normal porcine skin decorin DS	$5.7 \pm 0.5 \times 10^6$	$2.4 \pm 1.8 \times 10^{-3}$	0.18 ± 0.12	12.5×10^4
	Normal porcine skin biglycan DS	$4.44 \pm 0.07 \times 10^6$	$2.54 \pm 0.05 \times 10^{-2}$	6 ± 1.9	266×10^4

The interactions of immobilized DSs with various ligands were determined by the SPR method as detailed in *Materials and methods*. Interaction parameters were calculated using the non-linear curve fitting software supplied with the SPR instrument. Molecular masses of particular DS species, which are need to calculate the number of ligand-binding sites on 1 nM of GAG, were evaluated by comparison of these DS electrophoretic mobility with that showed by the standard GAGs. Obtained average Mr were as follows: Dupuytren's decorin DS 20 kDa, Dupuytren's biglycan DS 15 kDa, porcine decorin DS 25 kDa and porcine biglycan DS 22 kDa.

disaccharides. In contrast, DC biglycan DS has ~9% such units, while both porcine GAGs have only ~4%.

Competition assay included DC decorin DS interactions only with FGF-2 and FN since similar investigation we have previously performed for PDGF-BB (Koźma et al. 2009). The ability of IdoA or GlcA blocks to inhibit ligand binding to immobilized peptido-DS was conducted by the SPR method. Regions of both types were used in 10-fold excess when compared with mass immobilized onto a sensor chip. The data obtained are presented in Figure 6. As can be seen the type of hexuronate isomer is not crucial for DC decorin DS interactions both with FGF-2 and FN. Such a conclusion can be drawn from the observation that both GlcA and IdoA regions can efficiently inhibit each ligand binding to intact DS (Figure 6). Thus, our results suggest that DS sulfation can play a more important role for some interactions of the GAG than its glucuronosyl epimerization. However, statistical analysis of our data allowed to reveal a positive correlation only between 2,4-*O*-disulfated disaccharide content and DS affinity for PDGF-BB and between 2,6-*O*-disulafted disaccharide content and DS affinity for FGF-2. In addition, the former finding supports our previous observation (Koźma et al. 2009). On the other hand, in the case of DS interactions with FN, it can be concluded that the local assembly of appropriate sulfated disaccharides rather than their total content is responsible for the formation of high affinity binding site(s) in GAG chains.

Characterization of high-molecular-weight species existed in ECM of fibrous and normal fasciae

Our present SPR results suggest that DC-derived decorin and biglycan via their DS chains can form stable, frequently even irreversible links with some molecules such as FN, FGF-2 and PDGF-BB, which are also known to accumulate in ECM of disease-affected fascia. Thus, to confirm our in vitro observations concerning fascia DS binding abilities, we have assessed the composition of high-molecular-weight (HMW) components extractable with urea from DC-affected tissue. Previously, we have shown that these species before chondroitinase ABC treatment were in majority unable to penetrate even 3% stacking gel during sodium dodecyl sulfate (SDS)–PAGE (Koźma et al. 2005). Simultaneously, the enzyme generated several species with molecular weight >400 kDa and as the major product a component of electrophoretic mobility resembling that of both small DSPG core proteins (Koźma et al. 2005). However, in spite of enzyme digestion, a great part of HMW species still remained outside the preparative gel (Koźma et al. 2005). Now, we fractionated high molecular material extracted from DC fascia first by gel filtration on Sepharose CL-4B and then by chromatography on Sepharose CL-2B (Figure 7). From the latter bed, the species were eluted as a single broad and asymmetrical peak (Figure 7). Obtained fractions were collected as it is shown in Figure 7 and fraction components without or after chondroitinase ABC treatment were submitted to 4–15% gradient SDS–PAGE and

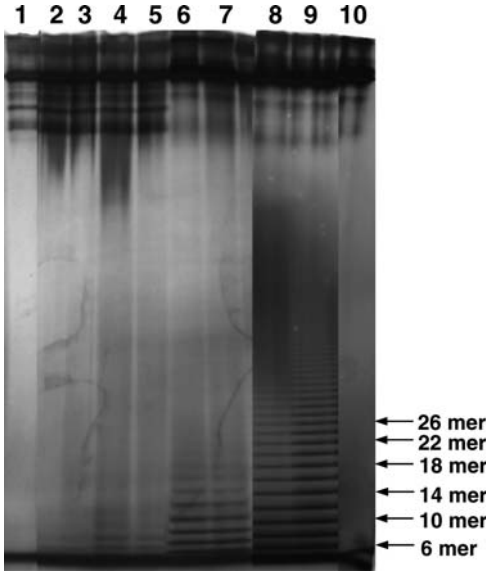

Fig. 4. Comparison of glucuronosyl epimerization patterns of DS chains from human fibrous fascia and normal porcine skin small PGs. DS chains were degraded by chondroitinase AC I or chondroitinase B action, and enzyme-resistant fragments were resolved by gradient PAGE followed by ammoniacal silver staining. Arrows show the migration position of DS oligosaccharide size markers with the most fast migrating hexasaccharide (6-mer). They were prepared by partial digestion of commercial DS by chondroitinase ABC followed by the gel filtration of degradation products on Bio Gel P-10 column (5 × 1000 mm) in 0.2 M NH_4HCO_3 at a flow rate of 1 mL/h. Lane 1, chondroitinase B preparation; lane 2, normal porcine skin decorin DS after chondroitinase B (GlcA blocks); lane 3, normal porcine skin biglycan DS after chondroitinase B (GlcA blocks); lane 4, fibrous human fascia decorin DS after chondroitinase B (GlcA blocks); lane 5, fibrous human fascia biglycan DS after chondroitinase B (GlcA blocks); lane 6, fibrous human fascia biglycan DS after chondroitinase AC I (IdoA blocks); lane 7, fibrous human fascia decorin DS after chondroitinase AC I (IdoA blocks); lane 8, normal porcine skin decorin DS after chondroitinase AC I (IdoA blocks); lane 9, normal porcine skin biglycan DS after chondroitinase AC I (IdoA blocks), lane 10, chondroitinase AC I preparation.

then stained with ammoniacal silver or probed with several antibodies. Electrophoretic profiles of both samples untreated with chondroitinase ABC and those digested with the enzyme resembled after silver staining (Figure 8A) the results obtained previously (Kośma et al. 2005) and described at the beginning of the section. Moreover, the present study allowed to reveal that these components of HMW species which penetrated to the preparative gel both before and after chondroitinase ABC digestion did not react neither with anti-versican, anti-FN, anti-PDGF-BB nor anti-FGF-2 sera (data not shown). In contrast, a strong reaction was observed solely for chondroitinase ABC-treated samples after the application of anti-biglycan and especially the anti-decorin serum (Figure 8B and C, respectively). The obtained immunoblots revealed the presence of individual DSPG core protein (Mr ~55 kDa) as the major product of enzyme action as well as series of slower migrating species (Figure 8B and C) which in the case of decorin displayed in majority higher molecular masses than intact PG (Figure 8B). In addition, significant similarity in respect of electrophoretic patterns is apparent between these immunoblots and silver-stained gels of HMW aggregates treated with chondroitinase ABC (Figure 8B and C vs A), suggesting that small DSPG core protein-like molecules are main species released from the above-mentioned aggregates by the enzyme. In turn, the lack within products of the chondroitinase ABC action of FGF-2, PDGF-BB or FN known to accumulate in fibrous fascia ECM indicates that these molecules may be strongly cross-linked forming visible previously supra-molecular complexes of molecular weight exceeding fractionation capability of the preparative gel (Kośma et al. 2005). On the other hand, the chondroitinase ABC ability to release from DC-derived HMW species remarkable amounts of small DSPG core protein-like supports the possibility that at least in part DS chains are responsible for very strong interactions of decorin (and biglycan) with other elements of heavy ECM aggregates. Thus, to identify the potential decorin DS-binding partners, intact DC-derived heavy aggregates were first immunoprecipitated with the anti-decorin serum and obtained material was then subjected not to SDS–PAGE but to dot blot followed by immunoreactivity assessment. As it results from Figure 9A, decorin containing HMW material from fibrous fascia ECM also displayed a positive and strong reaction with anti-PDGF-BB, anti-FGF-2 and especially with the anti-FN serum. Moreover, the presence of collagen type III as these HMW aggregate components were revealed (Figure 9A).

To further test whether decorin DS is able in vivo to direct bind the growth factor(s) and FN, we have examined the compositional alterations which affected heavy species isolated from ECM of normal fascia (NF) lata after chondroitinase ABC action. We used HMW species from the normal tissue due to their expected lower cross-linking degree as the result of lower tissue transglutaminase (TG) activity (for review, see Griffin and Bergamini 2002). In addition, we employed NF lata since this tissue material is more attainable than normal palmar fascia. Thus, NF ECM-derived heavy aggregates without or after chondroitinase ABC treatment were immunoprecipitated with anti-decorin antibody and then dot blotted and probed with several sera (Figure 9B). The NF aggregates that were deprived of DS/CS chains simultaneously failed to react with anti-FGF-2 and anti-PDGF-BB sera (Figure 9B). In addition, the effect observed was unconnected with a reduction in the ability of chondroitinase ABC-treated HMW material to immunoprecipitate with the anti-decorin serum. Such a conclusion can be drawn from approximated absorbance found for dot blots of the enzyme-treated NF aggregates which were subsequently immunoprecipitated with the anti-decorin or anti-FN serum and then probed with anti-FN antibody (Figure 9C). On the other hand, we observed that the removal of DS/CS chains intensifies the reaction of NF HMW species with anti-FN antibody (Figure 9B). Moreover, similarly as in the case of DC-derived species (Figure 9A), chondroitinase ABC treatment of NF-derived HMW aggregates leads to a reduction in their collagen type III content (Figure 9B).

To examine the sulfation pattern of DS/CS chains engaged in the formation of DC-derived supra-molecular complexes, the disaccharides obtained after these aggregate treatments with chondroitinase ABC were labeled with AMAC and

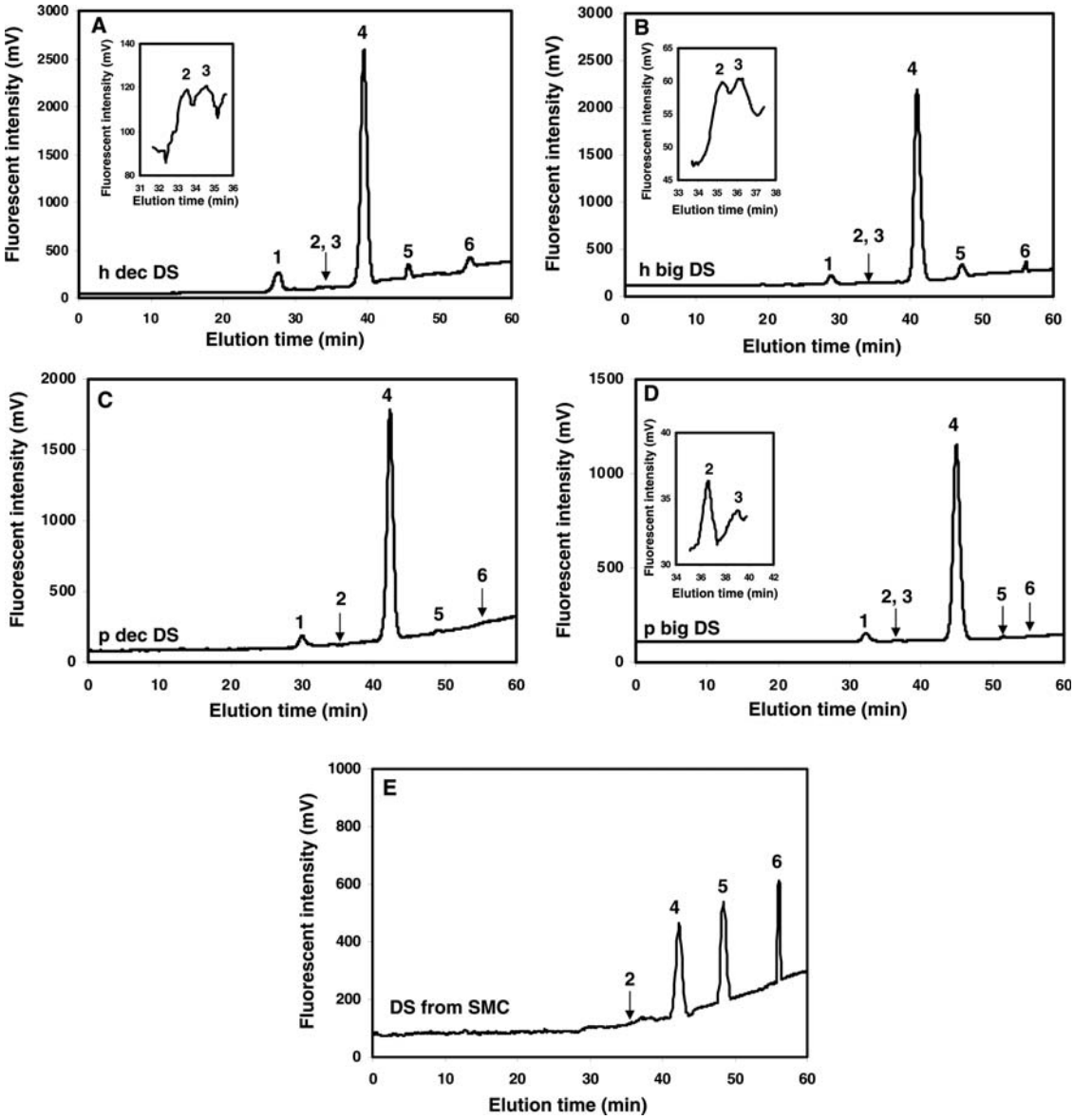

Fig. 5. Disaccharide analyses of DSs derived from small PGs (**A**–**D**) as well as from fibrous palmar fascia ECM supra-molecular complexes (**E**). DS chains were degraded by chondroitinase ABC. Obtained disaccharides after labeling with AMAC were resolved by RP HPLC and detected by fluorescence. Disaccharide peaks are numbered as follows: 1, ΔHexA(2S)-GalNAc(4S); 2, ΔHexA-GalNAc(4,6S); 3, ΔHexA(2S)-GalNAc(6S); 4, ΔHexA-GalNAc(4S); 5, ΔHexA-GalNAc (6S); 6, ΔHexA-GalNAc. (A) Disaccharide composition of fibrosis-affected human fascia decorin DS (h dec DS); (B) disaccharide composition of fibrosis-affected human fascia biglycan DS (h big DS); (C) disaccharide composition of normal porcine skin decorin DS (p dec DS); (D) disaccharide composition of normal porcine skin biglycan DS (p big DS); (E) disaccharide composition of fibrous fascia ECM supra-molecular complex DS (DS from SMC). Inserts in A, B and D show an increase in chromatogram area around 4,6-disulfate disaccharide peak (2) and 2,6-disulfate disaccharide peak (3).

submitted to RP HPLC (Figure 5E). As can be seen from Figure 5 and Table II, the enzyme-released disaccharide pool is completely deprived of 2,4-*O*-disulfated and 2,6-*O*-disulfated disaccharides and has reduced the content of 4-*O*-sulfated ones when compared with the sulfation pattern of DSs of decorin and biglycan extracted from DC as free molecules. On the other hand, higher content of 6-*O*-sulfated, 4,6-*O*-disulfated as well as unsulfated disaccharides is observed within the disaccharide pool rescued by chondroitinase ABC from DC ECM aggregates in comparison to DS chains of extractable decorin and biglycan.

Discussion

Our previous study has shown that when compared with urea extracts of the normal tissue containing mainly small DSPG decorin the advanced DC-affected fascia has remodeled the profile of extractable PGs (Koźma et al. 2005). The most prominent alteration reflects in high expression of another small DSPG—biglycan which in diseased tissue extracts was found in almost equimolar amounts with decorin (Koźma et al. 2005). The observed phenomenon leads to the substantial accumulation of DS in fibrosis-affected fascia. Moreover, the

Table II. Disaccharide composition of DSs from human Dupuytren's fascia and porcine skin small PGs as well as DS/CS from Dupuytren's fascia ECM supra-molecular complexes

GAG	ΔHexA (2S)-GalNAc (4S)	ΔHexA-GalNAc (4,6S)	ΔHexA (2S)-GalNAc (6S)	ΔHexA-GalNAc (4S)	ΔHexA-GalNAc (6S)	ΔHexA-GalNAc	Total sulfation (sulfates/100 disaccharide units)
Dupuytren's fascia decorin DS	7.59 ± 1.01	0.77 ± 0.15	0.22 ± 0.05	84.35 ± 1.61	5.31 ± 2.21	1.76 ± 0.49	107
Dupuytren's fascia biglycan DS	6.47 ± 1.55	0.4 ± 0.1	0.21 ± 0.08	89.47 ± 3.26	2.34 ± 1.85	1.12 ± 0.27	106
Normal porcine skin decorin DS	7.12 ± 1.67	0.19 ± 0.09	—	91.9 ± 1.37	0.7 ± 0.4	0.1 ± 0.02	107
Normal porcine skin biglycan DS	5.25 ± 0.89	0.3 ± 0.09	0.07 ± 0.01	93.94 ± 1.0	0.42 ± 0.12	0.07 ± 0.04	106
Dupuytren's fascia ECM supra-molecular complex DS/CS	—	1.03 ± 0.06	—	46.38 ± 1.33	38.48 ± 1.05	14.61 ± 1.63	87

Disaccharides obtained after degradation of DSs(/CS) by chondroitinase ABC were tagged with AMAC and resolved by RP HPLC as detailed in *Materials and methods*. Samples of individual GAG were analyzed at least in triplicate. Content of particular disaccharide is presented as average percentage ± SD of total disaccharide content.

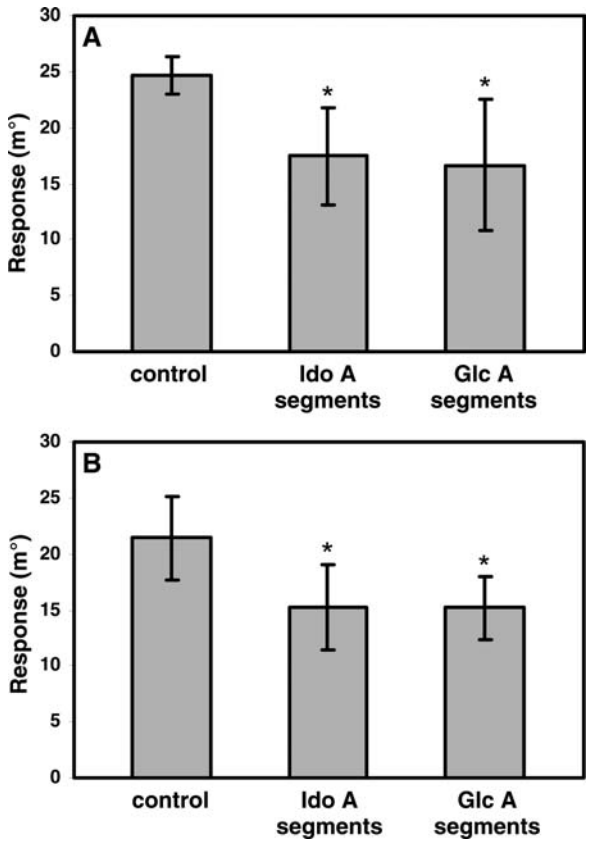

Fig. 6. Competition effects of IdoA and GlcA chain sections on FGF-2 (**A**) or FN (**B**) binding to DS chains. Biotinylated peptido-DSs of human fibrous fascia decorin were immobilized onto a streptavidin-coated sensor chip and exposed to individual ligand in the presence 10-fold excess of particular DS chain segments at physiological ionic strength. The binding was analyzed by plasmon surface resonance analysis as described in *Materials and methods*. Error bars show ligand binding as the mean ± SD of three experiments. Asterisks denote that differences are statistically significant ($P < 0.05$).

latter process is also associated with the structural remodeling of total tissue DS (Koźma et al. 2007). The alterations rather of quantitative nature manifest as an increase in the content of some IdoA and GlcA blocks, mainly these containing from 3 to 9 disaccharide units (Koźma et al. 2007). Moreover, such IdoA regions seem to include slightly more sulfate groups than appropriate segments present in NF DS chains (Koźma et al. 2007). An accumulation of structurally modified DS in DC fascia ECM arises the question concerning the role of the GAG in disease pathogenesis. Our present study allowed to reveal that decorin and biglycan DSs in fibrosis tissue can fulfill similar but not identical functions. Extractable pool decorin DS is able to bind most probably even in irreversible manner both growth factors (PDGF-BB and FGF-2) and FN as judged from very low K_d values characterizing all interactions. In turn, biglycan DS displays particularly high affinity to FGF-2, while it binds slightly more weakly remaining ligands. Interestingly, similar binding preferences were also found for normal porcine skin decorin and biglycan DSs suggesting that small DSPGs may have conserved functions in various tissues and circumstances. On the other hand, DS chains with high affinity to both growth factors and FN were rare in DC extracts that resulted in poor binding capacity of GAG-modified sensor chips during the SPR measurement. Most likely, in fibrosis-affected fascia, the DS with such binding properties is mainly engaged with the formation of supra-molecular complexes also containing PDGF, FGF-2 and FN. Such HMW aggregates just isolated by us from DC fascia ECM and characterized by immunological methods reveal remarkable compactness. This feature most probably is responsible for their insoluble character. Interestingly, we have previously found that about two-thirds of fibrous fascia DS pool remains unextractable (Koźma et al. 2007) supporting the possibility that the GAG contribution to the composition of ECM supra-molecular complexes may be substantial. However, unextractable fraction of tissue DS, albeit less abundant, also exists in NF (Koźma et al. 2007). Thus, the question arises concerning the role of DS in these ECM structures. Our present data suggest that DS forms a surface for strong molecular interactions which at least in part can stabilize ECM aggregates and influence their compactness. Such a conclusion can be drawn from a several of our present

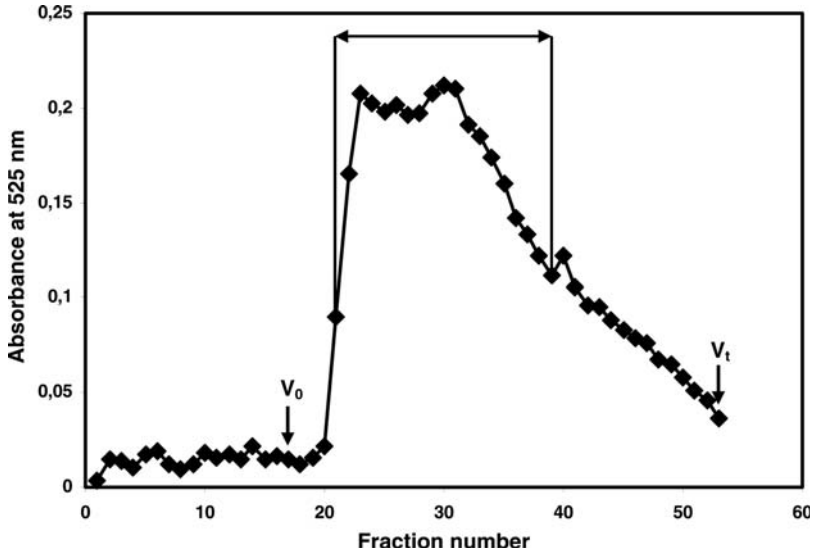

Fig. 7. Sepharose CL-2B gel chromatography of fibrous human fascia ECM-derived supra-molecular complexes containing PG(s). PG material eluted at void volume during gel chromatography on Sepharose CL-4B was submitted to chromatography on a Sepharose CL-2B column (1 × 44 cm) in 4 M guanidine HCl, 0.05 M sodium acetate, pH 5.8. Fractions of 0.5 mL were collected and monitored for GAGs. Fractions containing GAGs were pooled as indicated by bar. V_0 and V_t indicate the void volume and the total column volume, respectively.

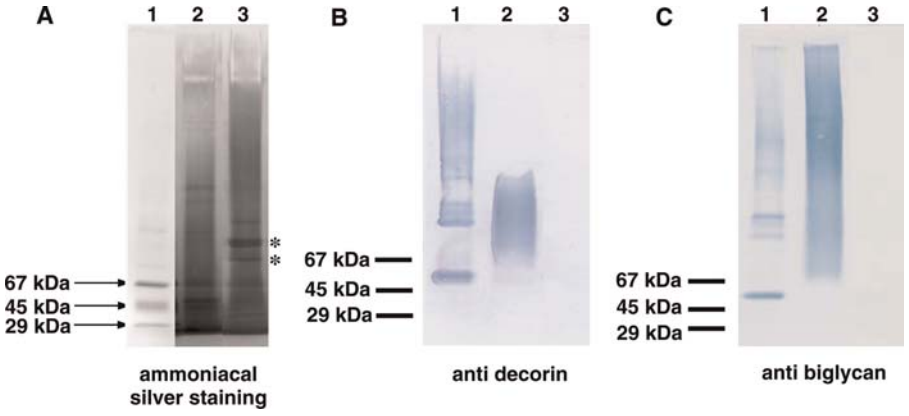

Fig. 8. Analysis of components released by chondroitinase ABC from HMW species of fibrosis-affected fascia ECM. HMW species extracted from DC fascia and purified by the gel chromatography set were without or after chondroitinase ABC treatment submitted to SDS–PAGE followed by ammoniacal silver staining (**A**) or western blotted and probed with the anti-decorin (**B**) or anti-biglycan (**C**) serum. The migration positions of the molecular weight markers are as indicated. (A) Lane 1, molecular mass markers: carbonic anhydrase (29 kDa), egg albumin (45 kDa) and bovine serum albumin (67 kDa); lane 2, HMW species untreated with enzyme; lane 3, HMW species after chondroitinase ABC action. Asterisks indicate the position of chondroitinase ABC-derived components. (B) Lane 1, anti-decorin immunoreactivity of HMW species after chondroitinase ABC action; lane 2, intact decorin of fibrosis-affected fascia; lane 3, anti-decorin immunoreactivity of HMW species untreated with chondroitinase ABC. (C) lane 1, anti-biglycan immunoreactivity of HMW species after chondroitinase ABC action; lane 2, intact biglycan of fibrosis-affected fascia; lane 3, anti-biglycan immunoreactivity of HMW species untreated with chondroitinase ABC.

observations. DS is responsible for direct, tight and stable binding of small PGs especially decorin to other components of ECM supra-molecular complexes. Our study has shown that partial destruction of the structure of fibrous fascia ECM heavy aggregates resulting in the abundant release of small DSPG core proteins is possible only when DS (and potentially CS) chains present within these heavy compounds are degraded. In addition, we have found a marked disproportion between significant GAG content in DC-derived HMW species, estimated by dimethylmethylene blue reaction (data not shown) and little sensitivity of these complexes to chondroitinase ABC. The observation suggests that particular DS/CS chains are poorly attainable for enzyme most probably due to simultaneous binding to several components of supra-molecular complexes. Further evidence for an important role of DS/CS for the structure of ECM-localized supra-molecular complexes comes from the analysis of such compounds derived from NF. Our examination of these aggregates has elucidated that their PDGF-BB and FGF-2 binding is almost completely dependent on DS/CS as judged from the

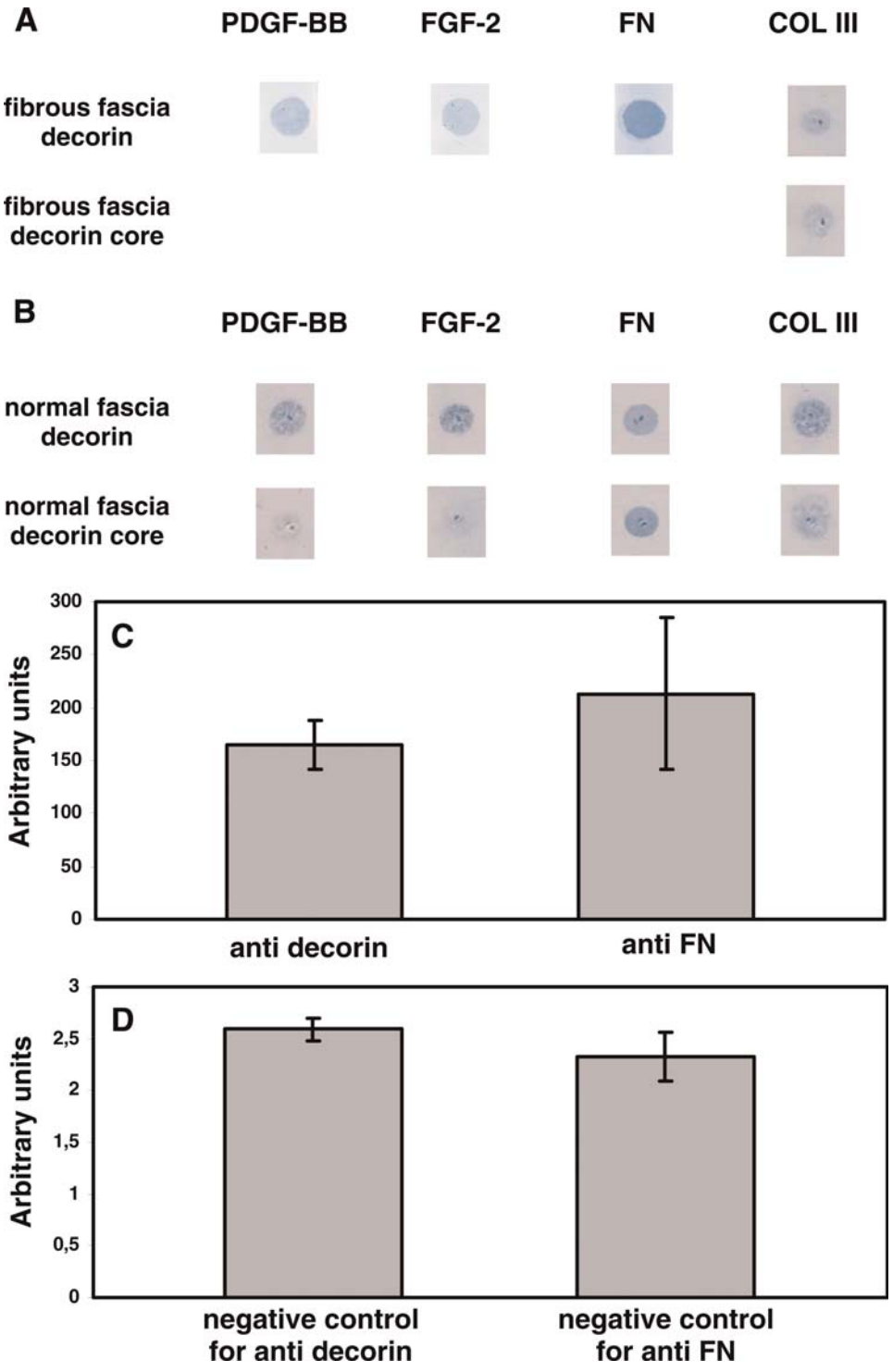

Fig. 9. Analysis of composition of supra-molecular complexes containing decorin. Multi-molecular complexes isolated from ECM of fibrous human fascia (**A**) or normal human fascia (**B**) were immunoprecipitated with the anti-decorin serum without and after chondroitinase ABC treatment. Precipitated compounds were dot blotted on Immobilon P and probed with several antisera as described in *Materials and methods*. (**C**) Quantitative comparison of immunoprecipitation of supra-molecular complexes deprived of DS/CS. NF ECM-derived supra-molecular complexes were treated with chondroitinase ABC and then immunoprecipitated with the anti-decorin or anti-FN serum. Precipitated compounds were dot blotted and probed with the anti-FN serum. (**D**) The impact of antibody present in samples of immunoprecipitated supra-molecular complexes on dot blot results. Immunoprecipitated species were dot blotted and treated directly with secondary antibody as described in *Materials and methods*. Error bars show the mean ± SD of the three experiments.

deterioration of aggregate immunoreactivity with anti-growth factor sera after the GAG depletion. This observation is concurrent with our previous finding that the destruction of DS/CS chains in fibrous fascia leads to the release of significant amount of species which reveal immunoreactivity with the anti-PDGF-BB serum (Koźma et al. 2009). Furthermore, it seems that DS/CS chains in ECM supra-molecular complexes are also at least in part able to direct bind FN molecules. This suggestion is supported by our present finding that DS/CS depletion is associated with a stronger reaction of NF HMW aggregates with the anti-FN serum most probably due to better epitope availability. In addition to DS/CS influence, the compactness of ECM supra-molecular complexes is most probably determined also by cross-linking of these aggregate components. This phenomenon seems to be particularly prominent in the case of fibrous fascia ECM aggregates and especially affects their FGF-2, PDGF-BB and FN components that reflected in the lack of such molecules among penetrating to the preparative gel species released by chondroitinase ABC. The cross-linking is most probably associated with the activity of Ca^{+2}-dependent TGs catalyzing the formation of γ-glutamyl--lysine cross-links (Griffin and Bergamini 2002). Interestingly, fibrosis process also that concerning palmar fascia is characterized by the enhanced activity of these enzymes (Dolynchuk and Pettigrew 1991). Among them, the most widespread TG type 2 (TG2) is mainly found on the cell surface and in ECM where it is colocalized with syndecan-4 and FN, respectively (Scrapellini et al. 2009). Moreover, cell surface heparan sulfate (HS) is able to bind strongly TG2 and to control the enzyme trafficking and activity (Scrapellini et al. 2009). Thus, taking into account that binding preferences of HS and DS frequently overlap, it is interesting whether the latter GAG apart from its direct impact on ECM aggregate compactness may also regulate this property in indirect fashion by influence on TG2 activity.

Sulfated GAGs interact with two binding sites on FN molecule which are designated as Hep-1 and Hep-2. The former one is connected with N-terminal type I repeats (Sekiguchi et al. 1983) and colocalized with FN-binding domain I_1–I_5 which contributes to the glycoprotein polymerization (Schwarzbauer 1991). Moreover, this site is cryptic in the intact folding FN molecules (Sekiguchi et al. 1983). The second GAG-binding region located near C-terminus (Lyon et al. 2000) contains three repeats of type III module (III_{12}–III_{14}) which are also essential for the FN fibril assembly as binding partners for domain I_1–I_5 (Bultmann et al. 1998). In addition, both decorin and biglycan core proteins interact with Hep-2 as well as with the cell-binding domain that is localized in Hep-2 proximity (Bidanset et al. 1992; Schaefer et al. 2003). Furthermore, the binding of the decorin core protein to FN is characterized by relatively high affinity (Bidanset et al. 1992), albeit lower than that found by us in the present study for DS interaction with FN. On the other hand, biological significance of GAG binding to FN is known particularly in the case of HS/heparin (H) (Woods et al. 1993). In turn, to date there is well evidenced the influence of decorin DS on cell adhesion/migration induced by FN (Bidanset et al. 1992; Merle et al. 1999). Our results obtained owing to the SPR method suggest that apart from the interaction mediated by their core proteins, small DSPGs can at physiological ionic strength strongly bind to FN via their GAG side chains. This two-point mode of small DSPG binding to FN as well as the overlapping of sites responsible on the FN molecules for both the protein polymerization and decorin binding suggest special importance of FN:DSPG interactions for some steps of FN fibrillogenesis. Thus, decorin (and most probably biglycan) after its binding to adjacent FN molecules could support FN unfolding as well as stabilize the FN–FN interactions by bridging effect. Interestingly, the expression of decorin in macrovascular endothelial cells, cultured on the collagen substratum, was associated with an induction of the FN matrix assembling (Kinsella et al. 2000). This finding however arises another possibility that decorin plays the role of factor connecting processes of collagen and FN fibrillogenesis. The hypothesis is also supported by several our present observations. As judged from immunoblots, some decorin core protein molecules released from DC-derived HMW species by chondroitinase ABC action are cross-linked with material which did not react neither with anti-FN nor with anti-growth factor sera. On the other hand, it is known that decorin core protein can bind to type III collagen (Thieszen and Rosenquist 1994) that is simultaneously substrate for TG (Griffin and Bergamini 2002). Interestingly, just we have found by the assessment of reactivity with the appropriate serum the presence of type III collagen both in DC and NF ECM-derived supra-molecular aggregates. Furthermore, dot blot analysis revealed that chondroitinase ABC treatment of these supra-molecular complexes also leads to a release of some portion of collagen type III. Thus, all above findings suggest tight relation between collagen type III and decorin core protein in both fibrous and NF. Moreover, taking into account that type III collagen is one of the known organizers of type I collagen fibrillogenesis (Birk and Mayne 1997), it is tempting to speculate that some fractions of fascia decorin can simultaneously bind type III collagen via core protein and FN via DS side chain contributing on this way to the regulation of the fibrillar matrix assembly. Interestingly, spatiotemporal convergence of type III collagen, FN and decorin expression is observed during the embryonic development of tendon (Kuo et al. 2008), which is an anatomical structure nearly related to fascia. Moreover, our present study has also shown that the ECM existence of supra-molecular aggregates involving both FN, decorin and collagen type III is common at least in some types of connective tissue independently of circumstances.

Our present results have confirmed our previous data that DS chains of both DC-derived small DSPGs display high affinity for PDGF-BB (Koźma et al. 2009). However, some discrepancy was found concerning K_d values characterizing especially DC decorin DS binding to the growth factor. This difference can result from the manner of DS immobilization which is crucial for GAG-binding properties (Osmond et al. 2002). It seems that in contrast to previously used solid-phase assay (Koźma et al. 2009), the SPR method with peptido-DS immobilized by its core protein fragment and GAG chain fully accessible to the growth factor allows to better estimate the GAG-binding potential.

The structural features of sulfated GAGs involved in their interactions with various bio-ligands are of special interest. Several studies, which however concern mainly HS/H interactions, allowed to reveal that these GAG binding to such molecules as FN or FGF-2 strongly depends on both overall sulfate density and appropriate sulfate group localization (Lyon et al. 2000; Kreuger et al. 2001). Moreover, minimal high-affinity binding regions in HS/H chains are formed by clusters of four disaccharides, two of which are at least disulfated (Lyon et al. 2000; Kreuger et al. 2001). In addition, highly sulfated fragments of HS/H chains contain exclusively IdoA residues which due to their conformational flexibility (Venkataraman et al. 1994) further promote these GAG ability to high-affinity binding. In contrast, structural requirement underlying DS interactions is less known. Our present results showing that the participation of IdoA residues is not essential to effective binding of DS to FN and FGF-2 are consistent with previous reports that both DS and CS can interact with the former ligand or promote the growth factor activity (Sekiguchi et al. 1983; Taylor et al. 2005; Ida et al. 2006). However, it seems that the binding capability of GlcA blocks more strongly depends on their sulfation pattern than in the case of IdoA blocks that reflect differences in conformational flexibility between both hexuronate isomers (Venkataraman et al. 1994). The excellent illustration of the hexuronate epimer impact on the binding properties is a finding that CS-E from squid cartilage containing more than 60% of GlcAβ1-3GalNAc(4,6S) disaccharides displays ~4-fold reduced affinity to FGF-2 when compared with CS–H from hagfish notochord which has remarkable amount of IdoAα1-3GalNAc(4,6S) (Deepa et al. 2002; Nandini et al. 2004).

Most likely, similarly as in the case of HS/H, DS/CS chain high-affinity binding sites for PDGF-BB monomer, FGF-2 and FN are also associated with rather short clusters also accumulating disulfated disaccharides. Our present study confirmed our previous observation (Koźma et al. 2009) that DS interaction with PDGF-BB can strongly depend on 2,4-*O*-disulfated disaccharide amount. In contrast, as it results from the report of Taylor et al. (2005), these disaccharides rather are not responsible for DS-dependent promotion of FGF-2 activity. However, higher affinity binding of the growth factor as well as effective promotion of its activity were found for DS chains containing 4,6-*O*-disulfated or 2,6-*O*-disulfated disaccharides in combination with 4-*O*-sulfated ones (Nandini et al. 2004; Taylor et al. 2005). Our present results are consistent with the above reports showing however the complexity of the problem. We observed a positive correlation between the content of 2,6-*O*-disulfated disaccharides in DS chains and the GAG affinity for FGF-2 simultaneously revealing that DS chains completely devoid of these disaccharides are still able to bind strongly the growth factor. Nevertheless, pivotal importance for DS/CS-mediated binding of PDGF-BB, FGF-2 and FN both of 2,6-*O*-disulfated and 2,4-*O*-disulfated disaccharides in addition to some 4-*O*-sulfated ones also results from our present finding that chondroitinase ABC is unable to release of these disaccharides from the GAG engaged with the structure of DC ECM supra-molecular complexes containing all above ligands. The possible explanation of the phenomenon is that DS/CS sections containing such disaccharides are inaccessible to enzyme due to their direct involving into interactions. Summarizing the above considerations concerning relation between DS/CS structure and functions, it may be concluded that, observed by us previously (Koźma et al. 2007) and mentioned at the beginning of this section, structural remodeling of total DS in fibrosis-affected palmar fascia can promote the formation of additional sites for strong ECM binding of FN, FGF-2 and PDGF-BB when compared with normal tissue. Mediated by fibrosis-affected fascia DS, the binding of the growth factors which precedes or even enables by proper positioning their cross-linking to other ECM components may represent the mechanism of the fibrosis process self-limitation. On the other hand, the immobilization of these most probably significant ECM deposits of growth factors as the result for example of Dupuytren's disease surgical treatment may have the clinical relevance underlying frequently observed incidents of pathological process recurrence.

Materials and methods

Reagents and materials

The following antibodies were used: monoclonal mouse anti-human decorin antibody (R&D Systems, Germany: no MAB 143), monoclonal mouse anti-human biglycan antibody 3E2 (Santa Cruz Biotechnology Inc., California; no sc-100857), polyclonal rabbit anti-human FGF-2 antibodies (PeproTech, UK; no 500-P18), polyclonal rabbit anti-human PDGF-BB antibodies (PeproTech; no 500-P47), monoclonal mouse anti-human FN antibody (QED Bioscience Inc., San Diego, California; no 42039), polyclonal rabbit anti-human versican V0/V2 antibodies (Abcam, UK; no ab 28671), polyclonal rabbit anti-human versican V0/V1 antibody (Sigma-Aldrich, Germany; no V5639), polyclonal rabbit anti-human collagen type III antibodies (Rockland, Gilbertsville, Pennsylvania; no 600-401-105-0.1), goat anti-rabbit immunoglobulin G conjugated to peroxidase and goat anti-mouse immunoglobulin G conjugated to peroxidase (Sigma-Aldrich). Urea, guanidine hydrochloride (GdnHCl), Sepharose CL-4B, chondroitinase ABC from *Proteus vulgaris*, chondroitinase B from *Flavobacterium heparinum*, peroxidase substrate 3,3′,5,5′-tetramethylbenzidine, papain, Tween-20, acrylamide, phosphate-buffered saline (PBS), 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate (CHAPS), AMAC, sodium cyanoborohydride, dimethyl sulfoxide (DMSO), ammonium acetate for HPLC, standard CS disaccharides (Δdi-0S sodium salt, Δdi-4S sodium salt, Δdi-6S sodium salt, Δdi-UA-2S sodium salt), standard DS from porcine intestinal mucosa, pronase, human plasma FN and protein G-Sepharose 4B fast flow were purchased from Sigma-Aldrich. Human recombinant PDGF-BB and FGF-2 were obtained from PeproTech. DEAE-Sephacel, Sepharose CL-2B and Octyl-Sepharose were supplied by Pharmacia Biotech (Sweden). Protease inhibitor cocktail [4-(2-aminoethyl)benzensulfonyl fluoride hydrochloride, aprotinin, bestatin, *N*-(trans-epoxysuccinyl)-L-leucine 4-guanidinobutylamide, leupeptin and pepstatin A], dimethylmethylene blue, tris (hydroxymethyl) aminomethane (Tris), molecular mass markers and *N*,*N*′-methylenebisacrylamide were obtained from Serva (Germany). Chondroitinase AC I from *F. heparinum* was supplied by Seikagaku (Japan). EZ-link sulfo-NHS-LC biotin

was purchased from Thermo Scientific, Rockford, Illinois. Standard CS 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, Hercules, California. Blot Quick Blocker was obtained from Chemicon International, Temecula, California. Remaining chemicals were supplied by POCH (Poland).

Tissue material

The samples of primary Dupuytren's fascia were obtained from 10 men (age range 45–65) treated operatively for the disease. Twelve specimens of NF lata were collected from healthy individuals of either sex (age range 50–71) during reconstruction surgery following accidental injury. All tissue specimens were obtained with informed consent of the patients. The study protocol was approved by the Regional Ethical Committee. Specimens of normal porcine skin were purchased from abattoir. Samples of particular fascia types were combined before preparation. All tissue samples were stored at −75°C until processing.

Isolation of decorin and biglycan from tissue samples

Both small DSPGs were isolated from degreased, dehydrated and homogenized tissue material as described previously (Koźma et al. 2005). Briefly, the tissue powder was extracted twice with 0.05 M sodium acetate, pH 6.0, containing 7.8 M urea, 0.2 M NaCl and protease inhibitors. Released ECM molecules were subjected to anion exchange chromatography on DEAE-Sephacel. PGs which adsorbed to the bed were subsequently eluted with buffered solution of 1 M NaCl and separated by gel chromatography on Sepharose CL-4B in respect of differences in their molecular weight. PGs running in the included volume were further fractionated by hydrophobic interaction chromatography on octyl-Sepharose CL-4B in 4 M GdnHCl. Under the used conditions, decorin molecules did not adsorb to octyl-Sepharose and were eluted from the column in the running buffer while biglycan ones bound to the bed were eluted with 1% CHAPS solution. The composition of obtained PG fractions was assessed by 4–15% gradient SDS–PAGE. The identity of DC-affected human fascia decorin and biglycan was also confirmed by the evaluation of fraction component immunoreactivity with antibodies recognizing the small DSPG core proteins. Moreover, the positive reactivity with above sera was also found for HMW species of PG origin which were extracted from fibrous fascia ECM and eluted at void volume during gel filtration on Sepharose CL-4B. These compounds as well as such species derived from NF lata were further purified by gel chromatography on Sepharose CL-2B in 0.05 M acetate buffer, pH 5.8, containing 4 M GdnHCl. Eluate fractions showing a positive reaction with dimethylmethylene blue (Farndale et al. 1986) were collected and characterized as described in the next sections.

Isolation of DS chains and their structural analysis

DS chains were released after pronase action on decorin and biglycan core proteins in 0.05 M Tris–HCl buffer, pH 7.5, at 30°C for 24 h at agitation. Then, enzyme was inactivated by boiling for 5 min and DS chains were subjected to DEAE-Sephacel chromatography in 0.05 M Tris–HCl buffer, pH 7.5. The GAG bound to the bed was desorbed with 1.5 M NaCl, dialyzed against distilled water and lyophilized.

To assess their glucuronosyl epimerization pattern, DS chains were partially depolymerized with chondroitinase AC I or B followed by the electrophoretic resolution of enzyme-resistant chain fragments on gradient PAGE as it was described previously (Koźma et al. 2005). To analyze the sulfation pattern, DS chains of particular small PGs as well as the GAG present in HMW species extracted from fibrous fascia ECM were first degraded by chondroitinase ABC in 0.05 M Tris–HCl buffer, pH 8.0, at 37°C for 24 h. Obtained unsaturated disaccharides were subsequently tagged with fluorophore AMAC and subjected to RP HPLC on Varian ProStar HPLC system according to Deakin and Lyon (2008). Briefly, lyophilized disaccharides were dissolved in 10 µL of 0.1 M AMAC in 85% DMSO/15% acetic acid and after 20 min treated with 10 µL of 1 M sodium cyanoborohydride. After 18 h incubation at room temperature, labeled disaccharides were diluted with the mixture of distilled water, DMSO and acetic acid (50:42.5:7.5) and applied to PRLP-S 300 A column (4.6 mm = 150 mm; Polymer Laboratories, Varian, Shropshire, UK) equilibrated in 0.1 M ammonium acetate. Then, column was washed with 2 mL gradient of 0–10% methanol. Adsorbed disaccharides were eluted with 50 mL linear gradient of 10–30% methanol and detected by in-line fluorescence (excitation at 425 nm, emission at 520 nm).

Biotinylation of decorin and biglycan DSs

Before the evaluation of their binding properties, DSs used as peptido-GAGs being products of papain action on small PGs (Koźma et al. 2009) were coupled on their peptide portion with biotin. To biotinylate, peptido-DSs (from 40 to 20 µg) were dissolved in 100 µL of 0.1 M PBS, pH 7.2, and incubated for 2 h at room temperature with 10 µL of 0.01 M EZ-Link Sulfo-NHS-LC-Biotin. Then, excess of biotin was removed by dialysis against distilled water and modified DSs were lyophilized. Efficiency of DS biotinylation was controlled with special EZ biotin quantitation kit (Thermo Scientific).

Analysis of DS interactions with growth factors and FN by the SPR method

The analysis was carried out in the Springle Instrument (Autolab, The Netherlands) employing biotinylated DS which was immobilized onto a streptavidin-coated sensor chip (SAHC 80 M from Xantec, Germany) and exposed to individual ligand used in five varying concentrations. The immobilization of DS chains was conducted in 0.04 M glycine/0.04 M NaCl solution, pH 3.0, for 10 min at 21°C. Subsequently, the chip was exhaustively washed with PBS, pH 7.4, containing 0.02% Tween-20 and blocked with biotin. Binding experiments were performed at physiological ionic strength in PBS, pH 7.4, containing 0.02% Tween-20, at 21°C until equilibrium was reached. The DS surface was regenerated between particular binding measurements by washing with 2 M NaCl in running buffer. Then, the dissociation of complex DS:ligand was measured only at the highest concentration of the latter

due to special properties of all investigated interactions which were characterized by high binding affinity and small binding capacity. The dissociation phase was generated by fast replacement of ligand solution with running buffer. Three independent sets of binding reactions and five replicates of dissociation phase for each ligand were performed. Parameters characterizing investigated binding reactions (i.e. association and dissociation rate constants k_a and k_d, respectively, equilibrium dissociation constant K_d as well as maximal binding capacity) were calculated for each set of binding curves using the nonlinear curve-fitting software supplied with the instrument.

Evaluation of GlcA or IdoA block ability to compete with intact DS for ligand binding

Contribution of particular DS chain sections in binding to PDGF-BB, FGF-2 and FN was analyzed by competition assay using the SPR method as described in the previous section. Briefly, biotinylated DC decorin peptido-DS chains were immobilized onto a streptavidin-coated sensor chip and exposed to single concentration of a particular ligand which was 15 min pre-incubated with or without 10-fold excess of DC decorin DS-derived IdoA or GlcA blocks. They were prepared by chondroitinase AC I or B, respectively, treatment of free GAG chains followed by the gel filtration of degradation products on Sephadex G-15 in 0.2 M NH_4HCO_3. Eluate was monitored by absorbance at 232 nm. Oligosaccharides eluted at void volume were collected, lyophilized and used to competition assay. The ability of particular chain section to inhibit ligand binding to intact DS was assessed as decrease in binding curve height.

Identification of fascia ECM-derived HMW species of PG origin

Two ways were applied to identify the composition of HMW aggregates. The first way was used for such species isolated from fibrous fascia ECM and purified by gel chromatography first on Sepharose CL-4B and then on Sepharose CL-2B. These species without or after previous chondroitinase ABC treatment were submitted to gradient 4–15% SDS–PAGE, electrotransfered to Immobilon P and then probed with anti-versican, anti-decorin, anti-biglycan, anti-FN, anti-PDGF-BB and anti-FGF-2 sera as described previously (Koźma et al. 2005, 2009). In addition to the above-mentioned treatment, HMW species derived from both fibrosis-affected and normal tissue were immunoprecipitated with the anti-decorin serum without or after prior chondroitinase ABC action. Immunoprecipitation was conducted in 0.02 M Tris–HCl buffer, pH 7.4, containing 0.3 M NaCl, 0.05% Triton X-100 as well as protease inhibitors for 12 h at 4°C, under agitation. Obtained immunological complexes were then incubated for 4 h with G-protein coupled to Sepharose beads. Components adsorbed to G-protein were solubilized in 0.25 M Tris–HCl buffer, pH 6.8, containing 4% SDS, by 5 min boiling. Positive control for immunoprecipitation was performed engaging used anti-decorin antibody and free human decorin. Precipitated material was then subjected to western blotting and probed with the same antibody.

Isolated by immunoprecipitation, decorins containing HMW aggregates due to their appreciable molecular masses exceeding fractionation ability of PAGE were further analyzed by dot blot according to protocol attainable on Abcam site. Briefly, aliquots of HMW species were blotted on Immobilon P previously prewet in methanol (15 s), deionized water (2 min) and dot blot buffer 0.02 M Tris–HCl, pH 7.5, containing 0.15 M NaCl, and 0.05% Tween-20 (5 min). The blots were then probed with anti-PDGF-BB, anti-FGF-2, anti-FN and anti-collagen type III sera. Due to the fact that samples of immunoprecipitated HMW species may contain unknown amount of used antibody (mouse anti-human decorin or in some cases mouse anti-human FN) which can influence final dot blot results, we performed a negative control for dot blot analysis. To this purpose, dot blotted samples of immunoprecipitated material were directly treated with dot blot secondary antibody (peroxidase conjugated rabbit anti-mouse IgG).

Quantitative assessment of PG samples

Protein content in PG samples was estimated by the Bradford (1976) method. In turn, GAG content was assessed by reaction with methyldimethylene blue (Farndale et al. 1986) as well as by an estimation of hexuronic acid content (Blumenkrantz and Asboe-Hansen 1973).

Statistical analysis

All experiments were undertaken at least three times unless specified. Data are presented as mean ± SD. Statistical evaluation was performed with the Student's *t*-test, accepting $P < 0.05$ as significant.

Funding

This work was supported by grant of Ministry of Science and Higher Education (NN 401232734) and by grant of Medical University of Silesia (KNW-1-101/09).

Conflict of interest statement

None declared.

Abbreviations

AMAC, 2-aminoacridone; CHAPS, 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate hydrate; CS, chondroitin sulfate; DC, Dupuytren's contracture; DMSO, dimethyl sulfoxide; DS, dermatan sulfate; ECM, extracellular matrix; FGF, fibroblast growth factor; FN, fibronectin; GAG, glycosaminoglycan; GalNAc, *N*-acetylgalactosamine; GdnHCl, guanidine hydrochloride; GlcA, glucuronic acid; ΔHexA, 4-deoxy-α-L-*threo*-hex-4-enepyranosyluric acid; HMW, high-molecular-weight; HS, heparan sulfate; IdoA, iduronic acid; NF, normal fascia; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PG, proteoglycan; RP HPLC, reverse-phase high-performance liquid chromatography; SDS, sodium dodecyl sulfate; SPR, surface plasmon resonance; TG, transglutaminase; Tris, tris(hydroxymethyl) aminomethane.

References

Ameye L, Aria D, Jepsen K, Oldberg A, Xu T, Young MF. 2002. Abnormal collagen fibrils in tendon of biglycan/fibromodulin-deficient mice lead to gait impairment, ectopic ossification, and osteoarthritis. *FASEB J*. 16:673–680.

Badalamente MA, Hurst LC. 1999. The biochemistry of Dupuytren's disease. *Hand Clin*. 15:35–42.

Baird KS, Crossan JF, Ralston SH. 1993. Abnormal growth factor and cytokine expression in Dupuytren's contracture. *J Clin Pathol*. 46: 425–428.

Bao X, Nishimura S, Mikami T, Yamada S, Itoh N, Sugahara K. 2004. Chondroitin sulfate/dermatan sulfate hybrid chains from embryonic pig brain, chich contain a higher proportion of L-iduronic acid than those from adult pig brain, exhibit neuritogenic and growth factor binding activities. *J Biol Chem*. 279:9765–9776.

Bidanset DJ, LeBaron R, Rosenberg L, Murphy-Ullrich JE, Hook M. 1992. Regulation of cell substrate adhesion: Effects of small galactosaminoglycan-containing proteoglycans. *J Cell Biol*. 118: 1523–1531.

Birk DE, Mayne R. 1997. Localization of collagen types I, III and V during tendon development. Changes in collagen types I and III are correlated with changes in fibril diameter. *Eur J Cell Biol*. 72:352–361.

Blumenkrantz N, Asboe-Hansen G. 1973. New method for determination of uronic acids. *Anal Biochem*. 54:484–489.

Bradford M. 1976. A rapid and sensitive method for the quatitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 72:248–254.

Bultmann H, Santas AJ, Pesciotta Peters DM. 1998. Fibronectin fibrillogenesis involves the heparin II binding domain of fibronectin. *J Biol Chem*. 273:2601–2609.

Cheng F, Heinegard D, Malmstrom A, Schmidtchen A, Yoshida K, Fransson LA. 1994. Patterns of uronosyl epimerization and 4-/6-*O*-sulphation in chondroitin/dermatan sulphate from decorin and biglycan of various bovine tissues. *Glycobiology*. 4:685–696.

Danielson KG, Baribault H, Holmes DF, Graham H, Kadler KE, Iozzo RV. 1997. Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility. *J Cell Biol*. 136:729–743.

Deakin JA, Lyon M. 2008. A simplified and sensitive fluorescent method for disaccharide analysis of both heparin sulfate and chondroitin/dermatan sulfates from biological samples. *Glycobiology*. 18:483–491.

Deepa SS, Umehara Y, Higashiyama S, Itoh N, Sugahara K. 2002. 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*. 277:43707–43716.

Dolynchuk KN, Pettigrew NM. 1991. Transglutaminase levels in Dupuytren's disease. *J Hand Surg Am*. 16:787–790.

Farndale RW, Buttle DJ, Barrett AJ. 1986. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim Biophys Acta*. 883:173–177.

Gonzalez AM, Buscaglia M, Fox R, Isacchi A, Sarmientos P, Farris J, Ong M, Martineau D, Lappi DA, Baird A. 1992. Basic fibroblast growth factor in Dupuytren's contracture. *Am J Pathol*. 141:663–671.

Griffin M, Bergamini CM. 2002. Transglutaminases: Nature's biological glues. *Biochem J*. 368:377–396.

Heldin CH, Westermark B. 1999. Mechanism of action and *in vivo* role of platelet-derived growth factor. *Physiol Rev*. 79:1283–1316.

Hikino M, Mikami T, Faissner A, Vilela-Silva ACES, Pavao MSG, Sugahara K. 2003. Oversulfated dermatan sulfate exhibits neurite outgrowth-promoting activity toward embryonic mouse hippocampal neurons. *J Biol Chem*. 278:43744–43754.

Ida M, Shuo T, Hirano K, Tokita Y, Nakanishi K, Matusi F, Aono S, Fujita H, Fujiwara Y, Kaji T, et al. 2006. Identification and functions of chondroitin sulfate in the milieu of neural stem cells. *J Biol Chem*. 281:5982–5991.

Kinsella MG, Fischer JW, Manson DP, Wight TN. 2000. Retrovirally mediated expression of decorin by macrovascular endothelial cells. Effects on cellular migration and fibronectin fibrillogenesis *in vitro*. *J Biol Chem*. 275:13924–13932.

Koshiishi I, Hasegawa T, Imanari T. 2002. Quantitative and qualitative alterations of chondroitin/dermatan sulfates accompanied by development of tubulointerstitial nephritis. *Arch Biochem Biophys*. 401:38–43.

Koźma EM, Głowacki A, Olczyk K, Ciecierska M. 2007. Dermatan sulfate remodeling associated with advanced Dupuytren's contracture. *Acta Biochim Pol*. 54:821–830.

Koźma EM, Olczyk K, Wisowski G, Głowacki A, Bobiński R. 2005. Alterations in the extracellular matrix proteoglycan profile in Dupuytren's contracture affect the palmar fascia. *J Biochem*. 137:468–476.

Koźma EM, Wisowski G, Olczyk K. 2009. Platelet derived growth factor BB is a ligand for dermatan sulfate chain(s) of small matrix proteoglycans from normal and fibrosis affected fascia. *Biochemie*. 91:1394–1404.

Kreuger J, Salmivirta M, Sturiale L, Gimenez-Gallego G, Lindahl U. 2001. Sequence analysis of heparan sulfate epitopes with graded affinities for fibroblast growth factors 1 and 2. *J Biol Chem*. 276:30744–30752.

Kuo CK, Petersen BC, Tuan RS. 2008. Spatiotemporal protein distribution of TGF-βs, their receptors, and extracellular matrix molecules during embryonic tendon development. *Dev Dynamics*. 237:1477–1489.

Laurent GJ, Chambers RC, Hill MR, McAnulty RJ. 2007. Regulation of matrix turnover: Fibroblast, forces, factors and fibrosis. *Biochem Soc Trans*. 35:647–651.

Lyon M, Deakin JA, Rahmoune H, Fernig DG, Nakamura T, Gallagher JT. 1998. Hepatocyte growth factor/scatter factor binds with high affinity to dermatan sulphate. *J Biol Chem*. 273:271–278.

Lyon M, Rushton G, Askari JA, Humphries MJ, Gallagher JT. 2000. Elucidation of the structural features of heparan sulfate important for interaction with the Hep-2 domain of fibronection. *J Biol Chem*. 275:4599–4606.

Merle B, Durussel L, Delmas PD, Clezardin P. 1999. Decorin inhibits cell migration through a process requiring its glycosaminoglycan side chain. *J Cell Biochem*. 75:538–546.

Nandini ChD, Itoh N, Sugahara K. 2005. Novel 70-kDa chondroitin sulfate/dermatan sulfate hybrid chains with a unique heterogenous sulfation pattern from shark skin, which exhibit neuritogenic activity and binding activities for growth factors and neurotrophic factors. *J Biol Chem*. 280:4058–4069.

Nandini ChD, Mikami T, Ohta M, Itoh N, Akiyama-Nambu F, Sugahara K. 2004. Structural and functional characterization of oversulfated chondroitin sulfate/dermatan sulfate hybrid chains from the notochord of hagfish. *J Biol Chem*. 279:50799–50809.

Osmond RIW, Kett WC, Skett SE, Coombe DR. 2002. Protein–heparin interactions measured by BIAcore 2000 are affected by the method of heparin immobilization. *Anal Biochem*. 310:199–207.

Prydz K, Dalen KT. 2000. Synthesis and sorting of proteoglycans. *J Cell Sci*. 113:193–205.

Rayan GM. 2007. Dupuytren's disease: Anatomy, pathology, presentation, and treatment. *J Bone Joint Surg Am*. 89:189–198.

Reinboth B, Hanssen E, Cleary EG, Gibson MA. 2002. Molecular interactions of biglycan and decorin with elastic fiber components. Biglycan forms a ternary complex with tropoelastin and microfibril-associated glycoprotein 1. *J Biol Chem*. 277:3950–3957.

Schaefer L, Beck KF, Raslik I, Walpen S, Mihalik D, Micegova M, Macakova K, Schonherr E, Seidler DG, Varga G, et al. 2003. Biglycan, a nitric oxide-regulated gene, affects adhesion, growth, and survival of mesangial cells. *J Biol Chem*. 278:26227–26237.

Schwarzbauer JE. 1991. Identification of the fibronectin sequences required for assembly of a fibrillar matrix. *J Cell Biol*. 113:1463–1473.

Scrapellini A, Germack R, Lortat-Jacob H, Muramatsu T, Billett E, Johnson T, Verderio EAM. 2009. Heparan sulfate proteoglycans are receptors for the cell-surface trafficking and biological activity of transglutaminase-2. *J Biol Chem*. 284:18411–18422.

Sekiguchi K, Hakomori S, Funahashi M, Matsumoto I, Seno N. 1983. Binding of fibronectin and its proteolytic fragments to glycosaminoglycans. Exposure of cryptic glycosaminoglycan-binding domains upon limited proteolysis. *J Biol Chem*. 258:14359–14365.

Sorensen V, Nilsen T, Więdłocha A. 2006. Functional diversity of FGF-2 isoforms by intracellular sorting. *BioEssays*. 28:504–514.

Sottile J, Hocking DC. 2002. Fibronectin polymerization regulates the composition and stability of extracellular matrix fibrils and cell-matrix adhesion. *Mol Biol Cell*. 13:3546–3559.

Taylor KR, Rudisill JA, Gallo RL. 2005. Structural and sequence motifs in dermatan sulfate for promoting fibroblast growth factor-2 (FGF-2) and FGF-7 activity. *J Biol Chem*. 280:5300–5306.

Terek RM, Jiranek WA, Goldberg MJ, Wolfe HJ, Alman BA. 1995. The expression of platelet-derived growth-factor gene in Dupuytren contracture. *J Bone Joint Surg Am*. 77:1–9.

Thieszen SL, Rosenquist TH. 1994. Expression of collagens and decorin during aortic arch artery development: Implications for matrix pattern formation. *Matrix Biol*. 14:573–582.

Tomasek JJ, Vaughan MB, Haaksma CJ. 1999. Cellular structure and biology of Dupuytren's disease. *Hand Clin*. 15:21–34.

Venkataraman G, Sasisekharan V, Cooney CL, Langer R, Sasisekharan R. 1994. A stereochemical approach to pyranose ring flexibility: Its implications for the conformation of dermatan sulphate. *Proc Natl Acad Sci USA*. 91:6171–6175.

Woods A, McCarthy JB, Furcht LT, Couchman JR. 1993. A synthetic peptide from the COOH-terminal heparin-binding domain of fibronectin promotes focal adhesion formation. *Mol Biol Cell*. 4:605–613.