Biochimie 91 (2009) 1394–1404

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Platelet derived growth factor BB is a ligand for dermatan sulfate chain(s) of small matrix proteoglycans from normal and fibrosis affected fascia

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ARTICLE INFO

Article history:
Received 27 April 2009
Accepted 16 July 2009
Available online 23 July 2009

Keywords:
Dermatan sulfate
Platelet derived growth factor
Normal fasciae
Dupuytren's fascia

ABSTRACT

Structural requirements of the short isoform of platelet derived growth factor BB (PDGF-BB) to bind dermatan sulfate (DS)/chondroitin sulfate (CS) are unknown. Meanwhile the interaction may be important for tissue repair and fibrosis which involve both high activity of PDGF-BB and matrix accumulation of DS. We examined by the solid phase assay the growth factor binding to DS chains of small proteoglycans from various fasciae as well as to standard CSs. Before the assay a structural analysis of DSs and CSs was accomplished involving the evaluation of their epimerization and/or sulfation patterns. In addition, in vivo acceptors for PDGF-BB in fibrosis affected fascia were detected. PDGF-BB binding sites on DSs/CSs are located in long chain sections with the same type of hexuronate isomer however without any apparent preference to glucuronate or iduronate residues. Alternatively, the interaction seems to involve two shorter DS chain sections assembling disaccharides with the same type of hexuronate isomer which are separated by disaccharide(s) with another hexuronate one. Moreover, DS/CS affinity to the growth factor most probably depends on an accumulation of di-2,4-O-sulfated disaccharides in binding site while the presence of 6-O-sulfated N-acetyl-galactosamine residues rather attenuates the binding. All examined fascia DSs and standard CSs showed significant PDGF-BB binding capability with the highest affinity found for normal palmar fascia decorin DS. In fibrosis affected palmar fascia DS/CS proteoglycans are able to form with PDGF-BB supramolecular complexes also including other matrix components such as type III collagen and fibronectin which bind the growth factor covalently. Our results suggest that DS chains of fascia matrix small PGs may regulate PDGF-BB availability leading to restriction of fibrosis associated with Dupuytren's disease or to control of normal fascia repair.

1. Introduction

Platelet derived growth factor BB (PDGF-BB) is a member of family mainly of homodimeric growth factors consisting of structurally related chains A–D linked by disulfide bonds (for review see [1–3]). The growth factor is secreted by various types of cells as a long variant also containing exon 6 encoded highly basic amino acid motif, so called retention region, localized near C terminus of each polypeptide chain. This motif can however be removed by proteolytic processing that occurs in extracellular space and leads to the PDGF-BB short version. Both forms of PDGF-BB display potentially the same biological effects mediated via the same set of tyrosine kinase receptors α and β (PDGFR α and β). Nevertheless, recently it has been reported that only long PDGF-BB is essential for proper vascular smooth muscle cells/pericytes assembly during vascular development [4].

Within broad repertoire of its biological functions PDGF-BB stimulates fibroblast proliferation, migration, and survival as well as collagen synthesis [1–3]. Thus the growth factor action is of special importance in the context of such events as wound repair and fibrosis. A paradigm of the last process is Dupuytren's disease classified as a palmar fascia fibromatosis [5]. This most probably inherited disease is characterized by the co-existence of two types of lesions: nodules comprising intensively proliferating (myo) fibroblasts and hypocellular pseudotendinous fibrous areas formed by fibrous cords [5]. The latter lesions representing advanced

Abbreviations: BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate hydrate; CS, chondroitin sulfate; DMSO, dimethyl sulfoxide; DF, Dupuytren's fascia; DS, dermatan sulfate; ΔHexA, 4,5-unsaturated hexuronic acid; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; GAG, glycosaminoglycan; GalNAc, N-acetyl-galactosamine; GlcA, glucuronate; HGF/SF, hepatocyte growth factor/scatter factor; HS, heparan sulfate; IdoA, iduronate; IgG, immunoglobulin G; K_d, dissociation constant; NFL, normal fascia lata; NPF, normal palmar fascia; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PDGF, platelet derived growth factor; PDGFR, platelet derived growth factor receptor; PG, proteoglycan; RP HPLC, reverse-phase high performance liquid chromatography; SDS, sodium dodecyl sulfate; TBS, Tris buffered saline; 2S, 2-O-sulfate; 4S, 4-O-sulfate; 6S, 6-O-sulfate.

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doi:10.1016/j.biochi.2009.07.010

disease stage lead to marked contracture of digits [5]. Moreover, disease affected fascia is also distinguished by both increased expression of PDGF B gene and strongly positive reaction for the growth factor antibodies as compared to normal tissue [6,7].

Activity of PDGF can be regulated at various levels some of which involve glycosaminoglycan (GAG) action. Heparan sulfate (HS) chains of cell and matrix proteoglycans (PGs) are able to attach long variant of PDGF-BB via interactions with the growth factor retention motifs [8]. The binding of long PDGF-BB engages HS regions containing at least ten disaccharide units enriched in N- and O-sulfated monosaccharide residues [9]. Associations of long PDGF with HS may protect the growth factor from proteolysis, concentrate active regulator near its cell receptors or induce establishment of ternary complexes with receptors as well as form storage reservoir of potentially active growth factor in extracellular matrix (ECM) [8,10]. Moreover, short variant of PDGF-BB initially considered as soluble and diffused form of the growth factor is able to form relatively stable complexes with heparin [10].

In addition to the PDGF-BB binding, GAGs can influence PDGFR activity. Upregulation of the growth factor receptors and an increase in their stability has been found in fetal lung fibroblasts growing in the presence of HS enriched in iduronate (IdoA) residues and sulfate groups [11]. Moreover, heparin and highly modified HS are also able to amplify PDGF-BB induced α and β receptor phosphorylation, respectively, as well as these receptor downstream signaling that reflects in higher migration response of cells [9,10]. In contrast, hyaluronan via its receptor CD 44 inhibits PDGFR β activation that results in disturbance of fibroblast migration [12].

However, surprisingly little is known about PDGF interactions with dermatan sulfate (DS) representing the major GAG component of matrix PGs. DS chains are composed of different number of disaccharide units containing N-acetyl-galactosamine residue usually existing as 4-O-sulfated derivative as well as glucuronate residue (GlcA) or IdoA residue being GlcA epimer frequently 2-O-sulfated [13]. IdoA residues determine DS chain flexibility while sulfate groups are engaged in ionic interactions of the GAG. Thus, variable contents of IdoA residues and sulfate groups reflecting differentiated extents of DS chain modifications may induce significant alterations in poorly known binding properties and biological functions of the GAG derived from different tissues or synthesized in the course of various processes, for instance fibrosis. In addition, in tissues undergoing fibrosis DS is abundantly deposited in ECM mainly as a component of two leucine rich PGs decorin and biglycan [14]. Taking into account that DS and PDGF-BB both are involved in fibrosis and DS can effectively compete with low-molecular heparin in short PDGF-BB variant binding [15] we have examined the capability of DSs derived from advanced Dupuytren's fascia decorin and biglycan to interact with the growth factor. Moreover, to assess poorly known structural requirements of small PDGF-BB variant to interact with DS/chondroitin sulfate (CS) we have also evaluated the growth factor binding to normal palmar fascia and normal fascia lata decorin GAGs as well as standard CSs.

2. Materials and methods

2.1. Reagents and materials

Urea, guanidine hydrochloride, Sepharose CL-4B, rabbit-anti-human biglycan core protein antibodies, chondroitinase ABC from *Proteus vulgaris*, chondroitinase B from *Flavobacterium heparinum*, heparinases I and III from *F. heparinum*, mouse monoclonal anti-human collagen type I IgG, mouse monoclonal anti-rabbit IgG conjugated to peroxidase, goat anti-mouse IgG conjugated to peroxidase, peroxidase substrate 3,3′,5,5′-tetramethylbenzidine, papain, standard C-6-S from shark cartilage, nitrocellulose membranes, Tween 20, acrylamide, standard collagen type I from calfskin, 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), 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 Sigma–Aldrich (USA). Human recombinant PDGF-BB and rabbit-anti-human recombinant PDGF-BB antibodies were obtained from PeproTech (UK). DEAE-Sephacel and Octyl-Sepharose were supplied by Pharmacia Biotech (Sweden). Dimethylmethylene blue, tris(hydroxymethyl)aminomethane (Tris), standard GAG (DS from porcine skin, C-4-S from whale cartilage, heparin), molecular mass markers, N,N′-methylenebisacrylamide were purchased from Serva (Germany). Chondroitinase AC I from *F. heparinum* was obtained from Seikagaku (Japan). Polyclonal rabbit-anti-human decorin core protein antibodies were supplied by Abcam (UK). Polyclonal rabbit-anti-human collagen type III antibodies were obtained from Rockland (USA). Monoclonal mouse anti-human fibronectin IgG was supplied by QED Bioscience Inc. (USA). 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, Bio-Gel P10 and Bio-Gel P6 were purchased from Bio-Rad Laboratories (USA). Blot Quick Blocker was obtained from Chemicon International (USA). Microtiter plates Immulon 2HB were purchased from Thermo Labsystems (USA). Remaining chemicals were supplied by POCH (Poland).

2.2. Tissue material

15 specimens of normal palmar fascia (NPF) and 10 samples of normal fascia lata (NFL) were collected from healthy individuals of either sex (age range 40–57 years) during reconstruction surgery following accidental injury. The samples of primary Dupuytren's fascia (DF) were obtained from 10 sex- and age matched patients, treated operatively for the disease. From each DF sample, the regions corresponding to fibrous lesions according to criteria set by Rombouts et al. [16] were collected. All tissue specimens were obtained with informed consent of the patients. The study protocol was approved by the Regional Ethical Committee. The samples, freed from adjacent tissues, were stored at −75 °C until processing.

2.3. Isolation and purification of decorin and biglycan

Both small matrix PGs were extracted and fractionated according to procedure described previously [14]. Briefly: PGs were extracted from homogenized, defatted and dehydrated tissue samples with buffered 7.8 M urea solution containing protease inhibitors. Then, both small PGs were purified from fibrillar proteins and hyaluronan as well as large-molecular weight PG(s) by anion exchange chromatography on DEAE-Sephacel and gel filtration on Sepharose CL-4B, respectively, and separated on the basis of differences in their core protein hydrophobicity by Octyl-Sepharose chromatography. The obtained decorin and biglycan fractions after the estimation of their protein and glycosaminoglycan content according to the methods of Bradford [17] and Farndale et al. [18], respectively, were further characterized by 4–15% polyacrylamide gradient gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) [14,19]. Moreover, the purity of each fraction was verified by Western immunoblotting with antibodies against

core proteins of human decorin and biglycan as described previously [14].

2.4. Isolation of small PG dermatan sulfate chains and evaluation of their structure

Before the DS chain isolation samples of particular small PG derived from each fascia type were combined in equal parts due to small amount of these molecules obtained especially in the case of NPF. Then, DS chains were released after digestion of decorin and biglycan core proteins with papain followed by elimination of residual core protein fragments by alkali treatment as described previously [14]. Next, free GAG chains were analyzed in respect of their molecular weight and glucuronosyl epimerization patterns. To attain this goal intact DS chains as well as products of their enzymatic degradation were submitted to gradient PAGE as described previously [14]. Two enzymes were employed for examination of DS chain composition: chondroitinase AC I that allowed to obtain clusters of IdoA disaccharides, and chondroitinase B that enabled to get GlcA disaccharide blocks. Obtained electrophoretic profiles were evaluated by densitometry. Before analysis of sulfation patterns fascia DSs as well as standard CSs were depolymerized by chondroitinase ABC in 0.05 M Tris–HCl buffer, pH 8.0, for 24 h at 37 °C and lyophilized. Dried disaccharides were further tagged with fluorophore 2-aminoacridone re-purified according to the method of Deakin and Lyon [20]. Disaccharide labeling with the fluorophore was conducted according to the method of Deakin and Lyon [20]. Briefly: disaccharides were dissolved in 10 μl of 0.1 M aminoacridone solution in 85% DMSO/15% acetic acid. After 20 min 10 μl of 1 M sodium cyanoborohydride was added to the disaccharide samples and they were incubated in the dark for 18 h at room temperature. Then, fluorophore labeled disaccharides were diluted with mixture of water and 85% DMSO/15% acetic acid (1:1) and subjected to reverse-phase high performance liquid chromatography (RP HPLC) according to Deakin and Lyon [20] on PLRP-S 300 Å column (4.6 mm × 150 mm; Polymer Laboratories, Varian, Shropshire, UK) equilibrated in 0.1 M ammonium acetate (solution A), running on a Varian ProStar HPLC system. After 2 ml gradient of 0–10% solution B (100% methanol), the disaccharides were eluted over 50 ml linear gradient of 10–30% solution B at a flow rate of 1 ml/min. Then, short and steep 3 ml gradient of 30–100% solution B was used. Disaccharides were detected by in-line fluorescence (excitation at 425 nm and emission at 520 nm). To evaluate the hexuronate composition of standard CSs, these GAGs were treated with chondroitinase AC I in 0.03 M Tris–HCl buffer, pH 7.3, for 3 h, at 37 °C. Then, obtained degradation products were submitted to chromatography on Bio-Gel P6 column (1 cm × 100 cm) in 0.1 M NH_4HCO_3. Fractions (1 ml) were eluted at a flow rate of 0.1 ml/min and analyzed by absorbance at 232 nm.

2.5. Analysis of DS–PDGF-BB interactions

To estimate the ability of DS chains to bind PDGF-BB the solid phase binding assay was used. In the assay palmar fascia DS chains were employed in amounts equivalent to 0.3 μg of PG core proteins while NFL decorin GAG chains were used in amount equivalent to 0.15 μg of PG core protein. All GAGs were solubilized in 100 μl of 0.01 M PBS, pH 7.4, containing 0.137 M NaCl and 0.0027 M KCl and added to microtiter plate wells. Well coating was conducted overnight at 4 °C and its efficiency was verified in a few wells by reaction with dimethylmethylene blue [18] on the adsorbed material. Upon coating wells were washed three times with 250 μl of PBS containing 0.05% Tween 20 (washing buffer) and incubated with 250 μl of 1% bovine serum albumin (BSA) in washing buffer for 1 h at room temperature to prevent nonspecific binding. After extensive rinsing with washing buffer the plates were incubated with 100 μl of washing buffer containing 1% BSA and various amounts of human recombinant PDGF-BB (short version) for 6 h at room temperature. Subsequently the wells were rinsed five times with washing buffer and incubated for 1 h at room temperature with washing buffer comprising 1% BSA and 0.1 μg of rabbit-anti-human recombinant PDGF-BB antibodies. After extensive rinsing the secondary antibody i.e. mouse monoclonal anti-rabbit IgG conjugated to peroxidase at a dilution of 1:50 000 (NFL DS) or 1:75 000 (palmar fascia DSs) with washing buffer containing 1% BSA was added and the plates were left for 1 h at room temperature. Then, after washing the colorimetric reaction was initiated by addition of 100 μl of peroxidase substrate 3,3′,5,5′-tetramethylbenzidine, and stopped after 30 min with 100 μl of 1 M HCl. Absorbance was measured at 450 nm using an ELISA microplate reader Asys Hitech (Biogenet). The above-mentioned procedure was also used to evaluate PDGF-BB binding to standard C-4-S from whale cartilage and C-6-S from shark cartilage. Both GAGs were employed in amounts approximated to fascia DSs (0.1 μg).

All samples were analyzed in five independent experiments. In parallel to test wells control ones were examined that were deprived of DS coat or PDGF. Moreover, for every experiment nonspecific binding was determined in the presence of 4.5 μg of heparin added together with 0.75 μg of PDGF to DS coated wells. The observed nonspecific binding was always negligible (average 5% of total binding).

To examine the contribution of GlcA and IdoA sections of DS chains to PDGF-BB binding the competition experiment was done. Wells coated with DF decorin DS were incubated with 0.75 μg of the growth factor and the GAG chain fragments generated by chondroitinase AC I or chondroitinase B. DF decorin DS degradation products of size larger than tetrasaccharides were obtained by gel filtration on Bio-Gel P10 in 0.2 M NH_4HCO_3 and in the competition experiment they were added to microtiter plate wells in five or ten fold excess in relation to the native DS amount.

2.6. Identification of glycosaminoglycan acceptors for PDGF-BB in Dupuytren's fascia

To assign the Dupuytren's contracture associated GAG acceptors for PDGF-BB, the growth factor was extracted from DF samples after enzymatic degradation of tissue CS/DS or HS. DF samples were initially extracted for 2 h at 25 °C in 0.02 M Tris–HCl buffer, pH 7.5, containing 4 mM $CaCl_2$ and protease inhibitor cocktail to remove potentially free PDGF. Then, growth factor extraction in the presence of chondroitinase ABC or heparinases I and III was conducted for 12 h at 37 °C or 25 °C, respectively. The former enzyme treatment was performed in 0.05 M Tris–HCl buffer pH 8.0, containing mixture of protease inhibitors, while the latter enzymes were applied in 0.02 M Tris–HCl buffer, pH 7.5, comprising 4 mM $CaCl_2$ and protease inhibitors. In parallel to enzyme treated DF samples the extraction only with appropriate buffers was done. All obtained extracts were collected by centrifugation. Subsequently, the extract components were precipitated overnight at 4 °C with ethanol, and after centrifugation they were subjected to 7.5–15% SDS-PAGE in the absence of reduction agents followed by electrotransfer to nitrocellulose membranes at conditions described previously [14]. Then, blots were extensively rinsed (3 × 5 min) in 0.05 M Tris–HCl buffer, pH 7.4, containing 0.15 M NaCl and 0.1% Tween 20 (TBS buffer). To block nonspecific antibody adsorption, the blots were incubated in TBS buffer containing 5% Blot Quick Blocker for 1 h at 21 °C. After washing with TBS buffer (3 × 5 min), the membranes were treated with polyclonal rabbit-anti-human recombinant PDGF-BB antibodies. These primary antibodies employed at concentration of 0.2 μg/1 ml of TBS, containing 5% Blot Quick

Blocker, were incubated with membranes for 1 h at 21 °C. Then, membranes were rinsed as above and treated for 1 h at 21 °C with peroxidase-conjugated mouse monoclonal anti-rabbit IgG, diluted 1:150 000 in TBS, containing 5% Blot Quick Blocker. Bands containing extracted PDGF-BB were visualized by using of peroxidase substrate 3,3′,5,5′-tetramethylbenzidine in solution. To identify ECM molecules linked to PDGF-BB, blots of extract components were also probed with anti-human type I collagen, anti-human type III collagen or anti-human fibronectin sera according to above described procedure.

2.7. 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 Duncan test accepting $p < 0.05$ as significant. Statistical evaluation of anti-human PDGF-BB serum immunoreactive component content in DF extracts was performed with the Student's t test, accepting $p < 0.05$ as significant.

3. Results

3.1. Structural characteristics of fascia small DSPG GAG chains submitted to PDGF-BB binding

Applied isolation and purification procedure allowed to obtain in the case of DF samples two relative abundant DSPG fractions: one containing decorin and the other comprising biglycan (Fig. 1C). In contrast, only the decorin fractions derived from both normal fasciae ensured sufficient amounts of material to further examinations (Fig. 1A and B). These results are consistent with our previous reports concerning small DSPGs of various types of fascia [14,19]. All obtained herein PG fractions manifested immunological homogeneity when probed with anti-decorin or anti-biglycan core protein antibodies (data not shown). Electrophoretic analysis of these PG fractions revealed that dependent on the fascia types decorin displays differential molecular masses (Fig. 2A). The apparent differences in respect of decorin molecular weight result exclusively from distinct size of DS chains modifying this PG (Fig. 2B and C). As can be seen in Fig. 2C, the GAG chains of decorin from DF and NFL showed approximated molecular weight ranging from 5 to 47°kDA and from 8 to 57 kDa, respectively, as compared to simultaneously electrophoresed reference DS. Instead, NPF decorin bears DS chains of distinctly lesser size ranging from 7 to 23 kDa.

Electrophoretic analysis of biglycan fractions confirmed our previous observation [14] that the appearance of two urea extractable biglycan species differentiated in respect of their electrophoretic mobilities is confined solely to DF samples (Fig. 3A). However, due to marked disproportion in content of both biglycan components that reflects in small amount of faster migrating PG isoform we did not separate its before DS chain isolation. DS chains of DF biglycan have smaller size than DF decorin ranging from 5.5 to 18 kDa (Fig. 3B).

An examination of various fascia DS structure included the evaluation both of glucuronosyl epimerization and sulfation patterns. The extent of the former modification was analyzed by gradient PAGE of chondroitinase AC I or B resistant DS chain fragments followed by densitometry of the obtained electrophoretic profiles. The quantitative analysis of glucuronosyl epimerization was accomplished on the two levels. On the first of them we estimated the average epimerization extent determined as the ratio of area under densitometric scans of chondroitinase AC I resistant chain fragments representing IdoA regions, and total area under densitometric scans of chondroitinase AC I and chondroitinase B

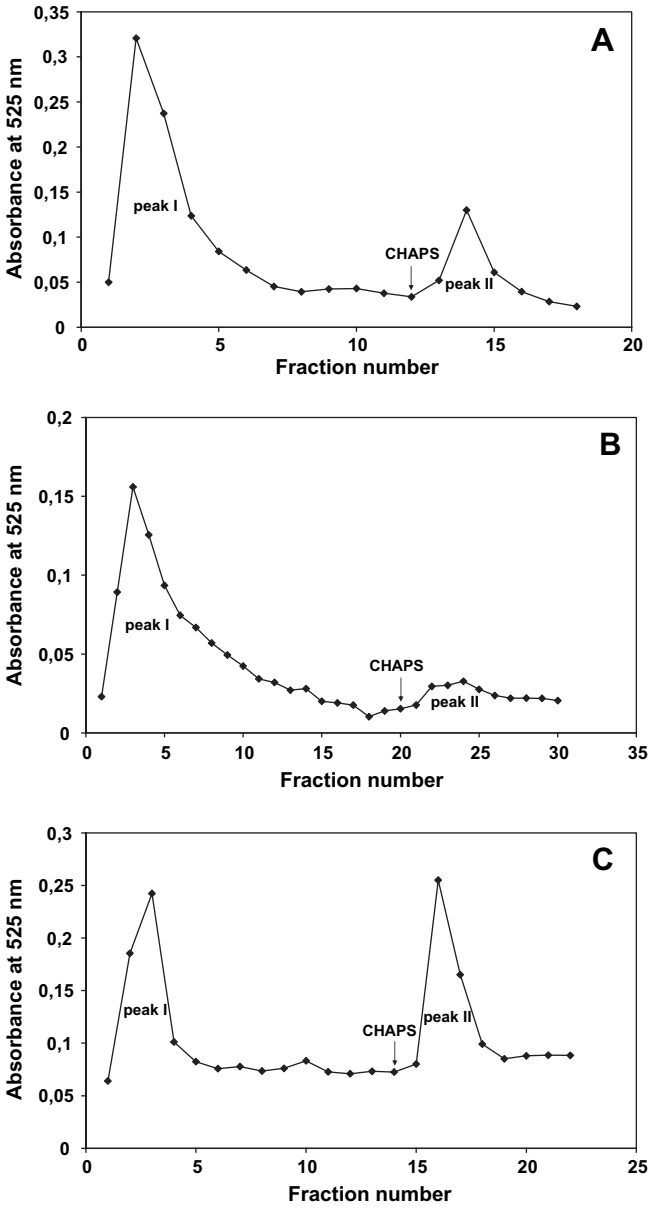

Fig. 1. Hydrophobic interaction chromatography of small DSPGs derived from NFL (A), NPF (B) and DF (C). Urea extractable small DSPGs were firstly purified by anion exchange chromatography and gel chromatography, and then separated by hydrophobic interaction chromatography. Unbound PG material contained decorin (peak I) while biglycan was stopped on the bed and eluted with CHAPS (peak II). The arrow indicates the appearance of CHAPS in the eluates.

resistant chain sections, representing IdoA and GlcA segments, respectively. However, it should be kept in mind that when staining with ammoniacal silver it is only possible to visualize chondroitinase resistant hexasaccharides or larger oligosaccharides [21]. Thus, epimerization extent calculated by us really reflects only predominant in DS "cluster epimerization" but it does allow for useful comparative analyses.

The second level of compositional analysis of various fascia DS chains included the evaluation of relative abundance of GlcA and IdoA chain sections with short and long size. We chose as the upper limit of short size DS chain regions the enzyme resistant oligosaccharides containing eight disaccharide units since it has been reported previously [9] that heparin species of such length yet displayed relatively little affinity for PDGF-BB. The presumable

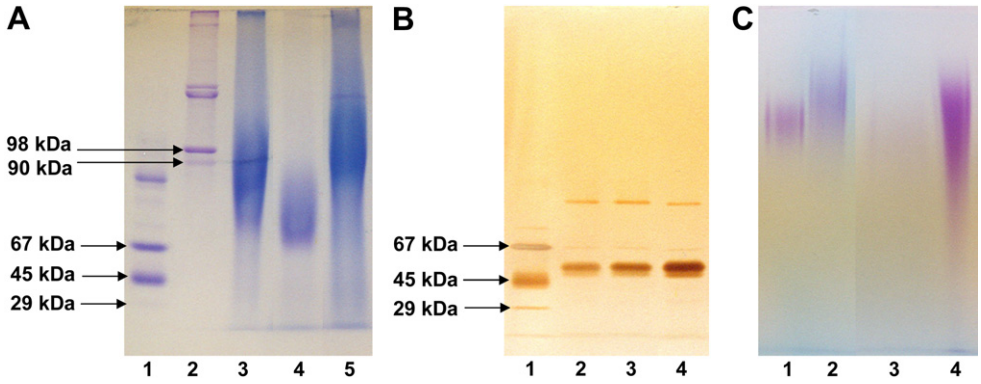

Fig. 2. Characteristics of various fascia decorin. (A) SDS-PAGE of decorin derived from NFL, NPF and DF. Decorin samples were resolved in a gradient gel and stained with Coomassie Blue. Lane 1, molecular mass markers (carbonic anhydrase, 29 kDa; egg albumin, 45 kDa; bovine serum albumin, 67 kDa). The apparent components of higher molecular weight represent most probably bovine serum albumin oligomers formed during storage of the mass marker solution; lane 2, standard collagen type I from calfskin; lane 3, decorin from NFL; lane 4, decorin from NPF; lane 5, decorin from DF. (B) SDS-PAGE of various fascia decorin core proteins. Decorin core proteins obtained after chondroitinase ABC treatment of PG were submitted to a gradient gel electrophoresis and stained with ammoniacal silver nitrate. Lane 1, molecular mass markers (carbonic anhydrase, 29 kDa; egg albumin, 45 kDa; bovine serum albumin, 67 kDa); lane 2, core protein of NFL decorin; lane 3, core protein of NPL decorin; lane 4, core protein of DF decorin. Bands of molecular weight $\geq$ 67 kDa derived from chondroitinase ABC preparation. (C) PAGE of various fascia decorin DS chains. DS chains obtained after papain digestion of decorin core proteins were electrophoresed in a gradient gels and stained with Azure A. Lane 1, standard DS of molecular masses 11–25 kDa; lane 2, DS of NFL decorin; lane 3, DS of NPF decorin; lane 4, DS of DF decorin.

polymerization degrees of particular DS oligosaccharides generated by chondroitinase AC I or B were calculated by comparison of electrophoretic mobilities of these degradation products and hexa- and octasaccharides obtained after complete cleavage of standard C-4-S by testicular hyaluronidase. Moreover, for DS oligosaccharide size calculation it was also taken into account that gradient PAGE separates GAG derived species depending on their molecular mass [21].

The obtained quantitative data (Fig. 4) concerning various fascia DS composition are in good agreement with our previous qualitative and semi-quantitative findings [14,22]. As it can be seen in Fig. 4A, all examined DSs display hybrid DS–CS structure albeit with significant contribution of GlcA disaccharide blocks. Moreover, composition of all examined fascia DSs is characterized by an assembly most of IdoA disaccharides into short chain sections (Fig. 4C). Nevertheless, distinct character of particular fascia DS epimerization patterns is evident reflecting in differential

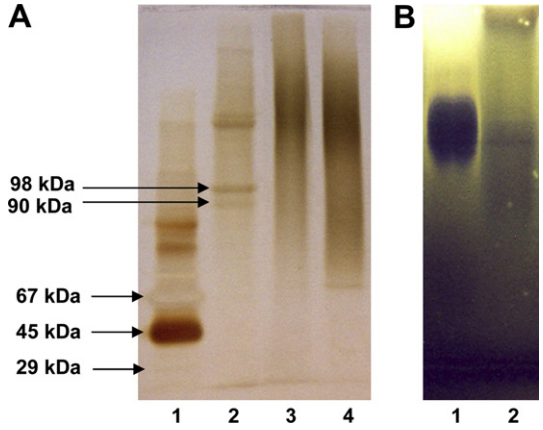

Fig. 3. Characteristics of DF biglycan. (A) SDS-PAGE of biglycan molecules derived from NFL and DF. Biglycan samples were resolved in a gradient gel and stained with ammoniacal silver nitrate. Lane 1, molecular mass markers (carbonic anhydrase, 29 kDa; egg albumin, 45 kDa; bovine serum albumin, 67 kDa); lane 2, standard collagen type I from calfskin; lane 3, biglycan from NFL; lane 4, biglycan from DF. (B) PAGE of DF biglycan DS chains. DS chains obtained after papain digestion of biglycan core protein were electrophoresed in a gradient gels and subjected to Azure A/ammoniacal silver nitrate staining. Lane 1, standard DS of molecular masses 11–25 kDa; lane 2, DS of DF biglycan.

contribution of GlcA and IdoA regions of different size in chain composition (Fig. 4B and C). Particularly dissimilar epimerization patterns were found for NPF decorin DS and DF biglycan DS. The former GAG reveals the highest content of long chain sections both IdoA and especially GlcA (Fig. 4B and C). In contrast, DF biglycan DS is characterized by the fewest contribution of long sections (Fig. 4B and C).

To examine fascia DS sulfation patterns unsaturated disaccharides obtained after GAG depolymerization with chondroitinase ABC were derivatized with fluorophore 2-aminoacridone and then subjected to RP HPLC. Typical chromatograms are presented in Fig. 5A–D. However, fluorescent peak areas differ from real content of particular disaccharides within a mixture [20]. Thus, the obtained results were corrected by multiplication of respective peak areas with appropriate experimental factors [20]. Data concerning relative contribution of individual disaccharides to various fascia DS structure are presented in Table 1. As can be seen from Table 1 all examined DSs display relatively similar average sulfate density. Moreover, more than 80% of these GAG disaccharides represent 4-O-sulfated ones. Nevertheless, individual character of each fascia DS sulfation pattern is apparent reflecting in differential content of other distinctly sulfated disaccharides since these minor components may confer special binding properties on mainly 4-O-sulfated fascia DS chains. In general, NPF decorin DS as compared to other examined GAGs is almost devoid of 6-O-sulfated disaccharides and has the highest content of unsulfated disaccharides (Table 1). On the other hand, NPF decorin DS comprises higher amount of di-2,4-O-sulfated disaccharides than both DSs from DF small PGs. However, the highest content of such disaccharides was found for NFL decorin DS (Table 1).

3.2. Evaluation of DS interactions with PDGF-BB

To assess the ability of various fascia DSs to bind human recombinant short PDGF-BB variant the solid phase assay with immobilized GAG and increasing amounts of the soluble growth factor was employed. In the experiment we used equal number of NPF and DF decorin DS chains as well as two fold reduced amount of NFL decorin DS and two fold increased amount of DF biglycan DS as compared to palmar fascia decorin GAGs. Such proportions of used GAGs were estimated in the preliminary experiment as optimal.

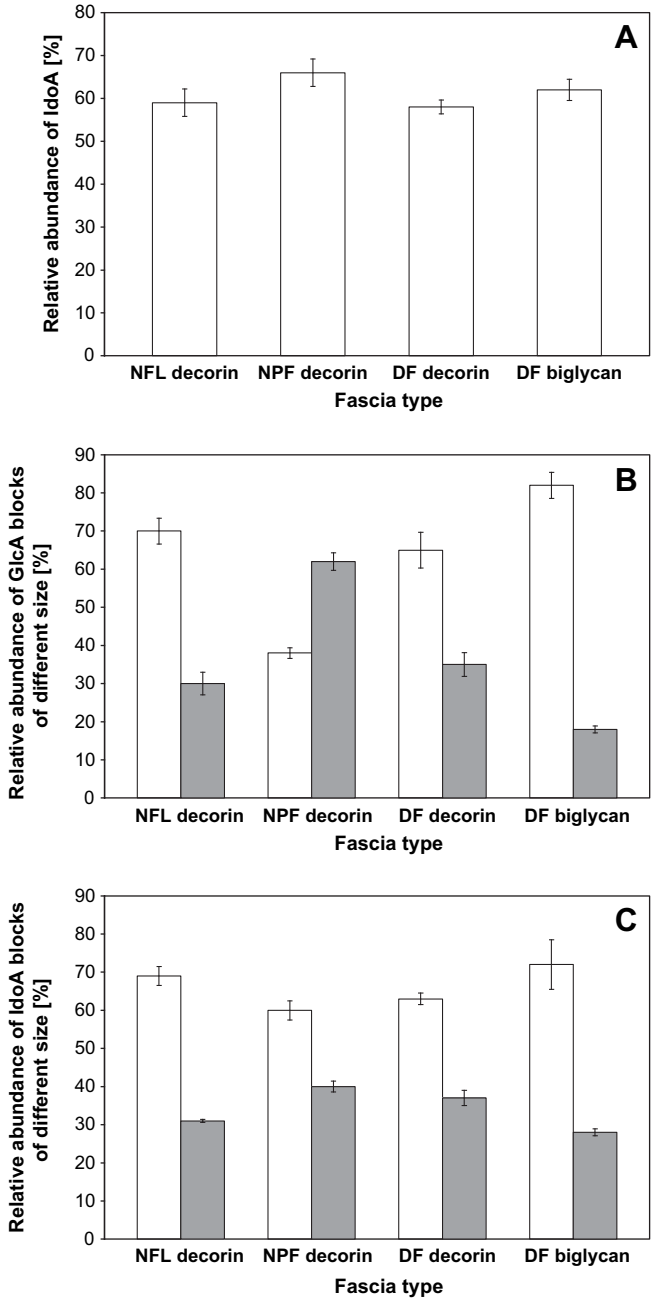

Fig. 4. Comparison of glucuronosyl epimerization patterns of DS chains from NFL decorin, NPF decorin, DF decorin and DF biglycan. (A) The relative epimerization extents of various fascia DSs calculated as the ratio of area under densitometric scans of chondroitinase AC I resistant chain fragment (IdoA region) electrophoretic profiles, and total area under densitometric scans of chondroitinase AC I and chondroitinase B resistant chain section (i.e. IdoA and GlcA segment, respectively) electrophoretic profiles. Error bars show the mean ± S.D. of the three experiments. Differences in the relative epimerization extents were statistically significant ($p < 0.05$) except those between NFL decorin DS and DF decorin DS. (B) The relative abundance of GlcA sections of short and long size in various fascia DS chains. As the upper limit of short size regions the chondroitinase B resistant oligosaccharides containing eight disaccharide units were chosen (see explanation in the Result section). Error bars show the mean ± S.D. of the three experiments. White bars – short size GlcA section contents, grey bars – long size GlcA section contents. (C) The relative abundance of IdoA sections of short and long size in various fascia DS chains. As the upper limit of short size regions the chondroitinase AC I resistant oligosaccharides containing eight disaccharide units were chosen (see explanation in the Result section). Error bars show the mean ± S.D. of the three experiments. White bars – short size IdoA section contents, grey bars – long size IdoA section contents.

As it was illustrated in Fig. 6A–D, PDGF-BB interactions with all examined DSs are dose-dependent and saturable. However, binding saturation was achieved for various DSs at different concentrations of the growth factor (Fig. 6A–D). The highest binding capacity was found for NFL decorin DS whereas DF biglycan DS bound the smallest amount of PDGF-BB taking into account variable number of used GAG chains. In addition, the binding capacity of fascia DSs showed good positive correlation with average molecular weight of their chains suggesting increased number of binding sites on longer GAG chains and as a consequence poor specificity of the interaction. In contrast, no linear correlation was found for the binding capacity of DSs and average extent of their "cluster" epimerization. However, further analysis revealed that the binding capacity of all palmar fascia (i.e. NPF and DF) DSs strongly depends on their contents of long sections both IdoA and GlcA. Then, to examine whether PDGF-BB binding sites on DS chains are associated with blocks of IdoA or GlcA disaccharides the competition experiment was carried out using DF decorin DS and products of its enzymatic degradation by chondroitinase AC I or chondroitinase B. The GAG was used due to its sufficient amount. The obtained results (Fig. 7) showed that both chondroitinase AC I and chondroitinase B resistant chain fragments effectively inhibit growth factor binding to native DS chains. However, slight differences in inhibition effect observed between GlcA and IdoA chain regions (Fig. 7) may arise from differences in their size and/or sulfation patterns. Moreover, the manifestation of binding inhibition only when ten fold excess of appropriate degradation products was used (Fig. 7) suggests that DF decorin DS contains few chain sections large enough to compete with native GAG for simultaneous binding to two sites on dimer ligand molecules.

On the basis of the binding saturation curves characterizing the interaction between short PDGF-BB isoform and various fascia DSs in the solid phase assay (Fig. 6A–D) the Scatchard-type plots were drawn according to Hedborn and Heinegard approach [23]. Data points obtained for each DS (Fig. 6A–D, insets) showed good correlation with straight line that suggests the presence of the single type PDGF-BB binding sites. Dissociation constant (K_d) values (Table 2) calculated on the basis of Scatchard graph equations indicate significant differences in various fascia DS affinity for the growth factor. The highest ability to bind the recombinant short PDGF-BB isoform was demonstrated by NPF decorin DS whereas NFL decorin DS displayed the lowest affinity to the growth factor. In turn, both DSs derived from DF small PGs show similar ability to bind PDGF-BB. On the other hand, the affinity of all examined DSs to the growth factor revealed good positive correlation with content of 4-O-sulfated disaccharides in these GAGs. In addition, the growth factor binding abilities of NPF and DF DSs were also strongly related with amount of di-2,4-O-sulfated disaccharides in these GAGs.

To further corroborate the significance of epimerization and sulfation patterns on GAG capability to interact with the small isoform of PDGF-BB we examined the growth factor binding to standard C-4-S and C-6-S. The experiment was preceded by structural analysis of these GAGs. Their sulfation patterns are presented in Table 1. Moreover, both GAGs displayed complete sensitivity to chondroitinase AC I (data not shown) which confirms the absence of IdoA residues. The binding abilities of standard CSs for PDGF-BB were estimated by the solid phase assay. Both GAGs were used for microplate well coating in approximate amounts as examined fascia DSs. Nevertheless, as can be seen in Fig. 6E and F both C-4-S and C-6-S displayed very high PDGF-BB binding capacity and we could not show any binding saturation at the growth factor concentrations sufficient to saturate fascia DS binding. However, likewise in the case of fascia DSs Scatchard plots obtained for each CS binding curve revealed one type of binding sites (Fig. 6E and F insets). Calculated K_d values (Table 2) indicated that C-4-S has

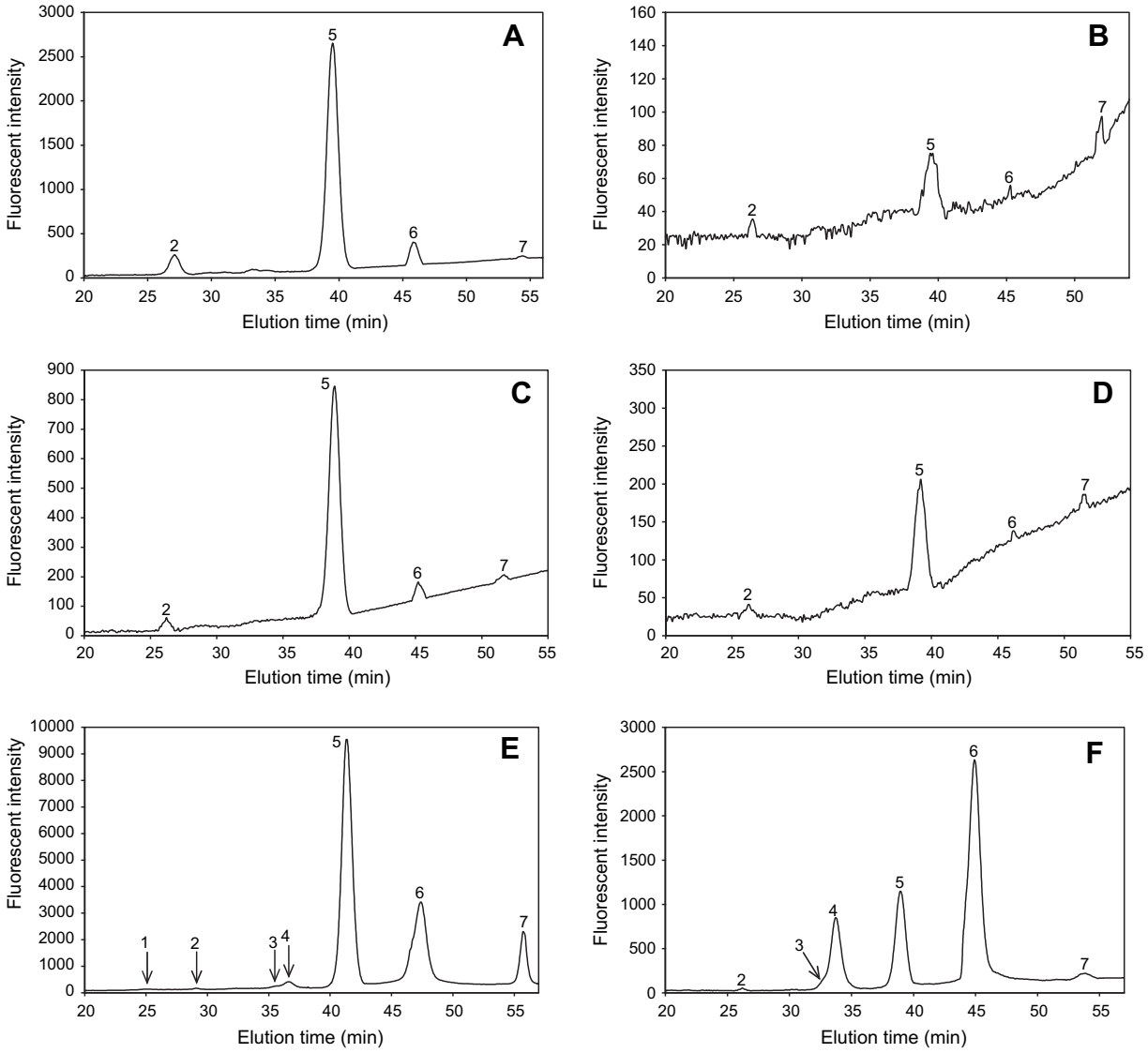

Fig. 5. Disaccharide analyses of DSs from various fascia small PGs and standard CSs. DSs released after proteolysis of core proteins and standard CSs were degraded by chondroitinase ABC. Obtained disaccharides were labeled with 2-aminoacridone, resolved by RP HPLC and detected by fluorescence. Disaccharide peaks are numbered as follows: 1, ΔHexA(2S)-GalNAc(4,6S); 2, ΔHexA(2S)-GalNAc(4S); 3, ΔHexA-GalNAc(4,6S); 4, ΔHexA(2S)-GalNAc(6S); 5, ΔHexA-GalNAc(4S); 6, ΔHexA-GalNAc(6S); 7, ΔHexA-GalNAc. (A) Disaccharide composition of NFL decorin DS; (B) disaccharide composition of NPF decorin DS; (C) disaccharide composition of DF decorin DS; (D) disaccharide composition of DF biglycan DS; (E) disaccharide composition of C-4-S from whale cartilage, arrows show positions of minor disaccharides; (F) disaccharide composition of C-6-S from shark cartilage, arrow show position of ΔHexA-GalNAc(4,6S).

Table 1
Disaccharide composition of various fascia DSs and standard CSs Disaccharides obtained after degradation of fascia DSs and standard CSs by chondroitinase ABC were tagged with 2-aminoacridone and resolved by RP HPLC as detailed under Materials and methods. Samples of individual GAG were analyzed at least in triplicate. Content of particular disaccharide is presented as average percentage ± S.D. of total disaccharide content.

GAG	ΔHexA(2S)-GalNAc(4,6S)	ΔHexA(2S)-GalNAc(4S)	ΔHexA-GalNAc(4,6S)	ΔHexA(2S)-GalNAc(6S)	ΔHexA-GalNAc(4S)	ΔHexA-GalNAc(6S)	ΔHexA-GalNAc	Total sulfation (sulfates/100 disaccharides)
NFL decorin DS	–	9.25 ± 0.84	–	–	84.12 ± 0.1	4.79 ± 0.63	1.84 ± 1.6	107
NPF decorin DS	–	6.97 ± 0.02	–	–	86.96 ± 0.1	0.35 ± 0.14	5.72 ± 0.4	101
DF decorin DS	–	6.44 ± 0.54	–	–	86.58 ± 3.3	5.01 ± 2.7	1.93 ± 0.03	104
DF biglycan DS	–	6.55 ± 2.01	–	–	88.44 ± 0.01	1.99 ± 0.52	3.02 ± 2.5	104
C-4-S from whale cartilage	0.24 ± 0.05	0.11 ± 0.04	0.49 ± 0.12	1.38 ± 0.24	77.15 ± 2.81	18.63 ± 2.45	2.0 ± 0.42	100
C-6-S from shark cartilage	–	4.92 ± 1.87	1.4 ± 0.57	14.16 ± 3.20	28.71 ± 6.8	47.73 ± 9.5	3.08 ± 2	117

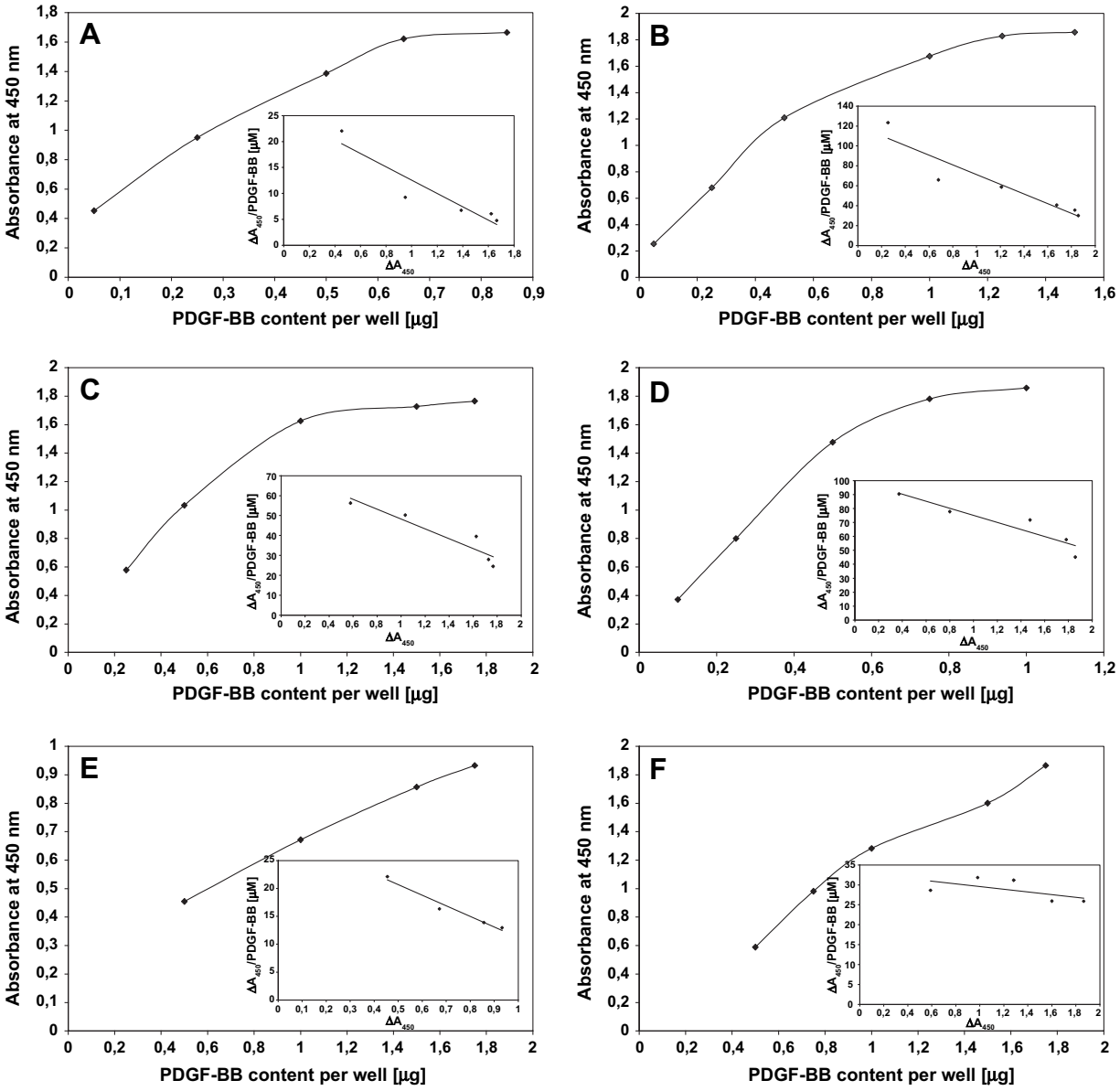

Fig. 6. Saturation binding of recombinant human PDGF-BB to DS of NFL decorin (A), DS of NPF decorin (B), DS of DF decorin (C), DS of DF biglycan (D), standard C-4-S from whale cartilage (E) and standard C-6-S from shark cartilage (F). Immobilized GAGs were incubated with increasing amounts of PDGF-BB. The growth factor binding was determined by using of rabbit-anti-human recombinant PDGF-BB antibodies as well as peroxidase-conjugated mouse anti-rabbit IgG followed by colorimetric reaction. PDGF-BB binding to particular GAG was analyzed in five experiments. Results of one representative experiment for each GAG are shown. The insets show a Scatchard-type plot of the experimental data for PDGF-BB binding to individual GAG.

significantly higher affinity for PDGF-BB than C-6-S. Moreover, the former GAG shows growth factor binding capability resembling that of DSs derived from both DF small PGs.

For evidence that fascia GAGs are in vivo acceptors for PDGF-BB, tissue samples were extracted with or without GAG depolymerizing enzymes (chondroitinase ABC or heparinases I and III), and extract components were further analyzed by Western blotting with anti-human PDGF-BB polyclonal antibodies. In this experiment we have employed only DF samples since both NPF and NFL are known to express very low level of the growth factor [6,7]. Densitometric analysis of the obtained immunoblots (Fig. 8A,B) unequivocally indicates that only the treatment of DF with chondroitinase ABC markedly increases the release of heterogenous species demonstrating immunoreactivity with anti-PDGF-BB serum. Moreover, the apparent enzyme effect can be affected by pH dependent differences in extract component solubility (Fig. 8A,B). All anti-PDGF-BB serum reactive components of DF extracts obtained after chondroitinase ABC action showed relatively high-molecular masses up to 120 kDa (Fig. 8A). In contrast, negligible amounts of low-molecular weight species corresponding to short form of the growth factor were found only in pH 7.5 extracts with or without heparinases (Fig. 8A). On the other hand, large size of chondroitinase ABC extract components displaying anti-PDGF-BB immunoreactivity as well as their resistance to thermal denaturation in the presence of SDS indicated that they may represent molecular complexes containing PDGF-BB covalently linked most probably to fragments of ECM molecules. To examine the nature of the latter components the blots of species released from DF by chondroitinase ABC were probed with anti-collagen type I, anti-collagen type III or anti-fibronectin sera, respectively. The obtained

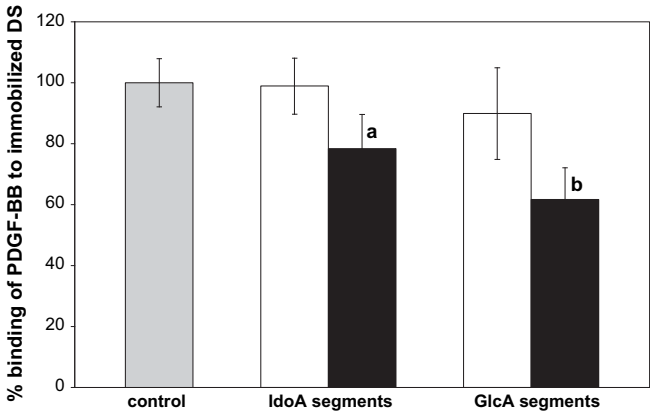

Fig. 7. Competition effects of IdoA and GlcA chain sections on PDGF-BB binding to DS chains. Immobilized native DS chains of DF decorin were incubated with PDGF-BB and five (white bars) or ten (black bars) fold excess of the GAG chain fragments generated by chondroitinase AC I or chondroitinase B. The growth factor binding was determined by using of rabbit-anti-human recombinant PDGF-BB antibodies as well as peroxidase-conjugated mouse anti-rabbit IgG followed by colorimetric reaction. Error bars show the relative PDGF-BB binding as the mean ± S.D. of three experiments. a – differences statistically significant ($p < 0.05$) in relation to PDGF-BB binding to DF decorin DS in the absence of the DS chain fragments (control). b – differences statistically significant ($p < 0.01$) in relation to PDGF-BB binding to DF decorin DS in the absence of the DS chain fragments (control).

results (Fig. 8C–E) indicate that both blots treated with anti-human fibronectin and anti-human collagen type III antibodies revealed similar profile as that observed for blots probed with anti-PDGF-BB serum (Fig. 8). However, further investigations are required to assign the exact composition of supramolecular complexes including PDGF-BB in DF ECM.

4. Discussion

Our present data confirm previous report [15] that DS/CS can strongly bind short PDGF-BB variant which was initially considered as soluble and diffusible form of the growth factor [8]. Moreover, our results indicate that the interaction is rather poorly specific as judged from appreciable binding capacity showed by structurally distinct GAGs. We have also attempted to our knowledge for the first time to evaluate structural features of DS/CS chains which promote the small PDGF-BB variant binding. It seems that the interaction is unaffected by the type of hexuronate isomer in binding site as judged from similar effectiveness of chondroitinase B and chondroitinase AC I resistant chain fragments to inhibit PDGF-BB binding to native DF decorin DS. This lack of clear preference for specific hexuronate isomer also characterizes the interactions of the growth factor long variant with HS [9]. In contrast, other growth factors as hepatocyte growth factor/scatter factor (HGF/SF) or some members of fibroblast growth factor family more strongly interact with GAG sequences abundant in IdoA residues [24,25].

Table 2
Dissociation constants (K_d) for binding of PDGF-BB to various fascia DSs and standard CSs Increasing amounts of PDGF-BB were incubated with the individual immobilized GAG as shown for Fig. 5, and the K_d values were calculated by Scatchard analysis. Data are mean values ± S.D. at least of three analyses. Differences were statistically significant ($p < 0.05$) except those between DF decorin DS, DF biglycan DS and C-4-%.

GAG type	K_d [nM]
NFL decorin DS	91.58 ± 12.7
NPF decorin DS	15.72 ± 4.3
DF decorin DS	38.94 ± 5.6
DF biglycan DS	36.94 ± 13.1
C-4-S from whale cartilage	52.3 ± 8.4
C-6-S from shark cartilage	299.6 ± 19.7

Our present results also suggest the existence of two types of PDGF-BB binding site arrangement in GAG chains. In the first type of binding site disaccharides with the same hexuronate isomer are assembled into single long section. Such binding sites exist besides CSs also in DS chains as judged from the ability of both chondroitinase AC I and chondroitinase B resistant fragments of DF decorin DS to inhibit dimeric ligand binding to native GAG. Alternatively, PDGF-BB recognition site on DS chains may be composed of two shorter clusters of disaccharides with the same type of hexuronate isomer which are separated by disaccharide(s) with opposite hexuronate one. The former regions could be responsible for direct binding to each PDGF-BB monomer. Such binding sites most probably more frequent than those of the first type should be however disrupted by bacterial lyases. These properties of the second type binding sites could explain our observation that chondroitinase AC I and chondroitinase B resistant fragments of DF decorin DS were not as effective as native chains in binding to dimeric ligand. Interestingly, above-mentioned structure of binding site on GAG chain is also postulated in the case of HS interacting with long PDGF-BB variant [9].

Our present data also suggest that DS/CS binding to the short PDGF-BB involves 4-O-sulfated disaccharides and especially di-2,4-O-sulfated ones. However, the suitable localization or local accumulation of the latter disaccharides rather than their total content influences binding capability of DS/CS. Such a conclusion can be drawn from the fact that NFL decorin DS distinguished among all examined fascia DSs by the highest amount of di-2,4-O-sulfated disaccharides simultaneously displayed the lowest affinity for PDGF-BB. Moreover, standard C-4-S which showed the lowest content of these disaccharides within all GAGs examined by us, demonstrated higher capability to bind PDGF-BB than NFL decorin DS. In addition, local accumulation of negative charge in NPF decorin DS chains may be responsible for the formation of high affinity binding site(s) for PDGF-BB in the GAG. Such a possibility results from our previous observation [14] that IdoA sections of the GAG revealed remarkable heterogeneity in respect of electrophoretic mobility of the same length segments that suggests significant differences in their sulfation density. Thus, the DS/CS affinity for the short PDGF-BB variant may depend on sulfate density as in the case of other growth factors as large PDGF-BB and HGF/SF [9,26]. However, from our study also results that unlike to positive effect of 4-O-sulfated GalNAc on short PDGF-BB binding to CS/DS, 6-O-sulfated hexosamine residues seem to be less preferable for the interaction. Such a conclusion may be drawn from the finding that the affinity of all examined GAGs for PDGF-BB strongly negative correlates with their 6-O-sulfate disaccharide content. The phenomenon can be explained by the fact that 6-O-sulfated GalNAc residues are concomitant with GlcA ones [27] which are characterized by conformational rigidity. The latter property may lead to discriminate or hinder the accommodation of 6-O-sulfated disaccharide containing/clustering regions of CS/DS to binding site on short PDGF-BB variant molecules. In contrast, 6-O-sulfate groups in HS seem to be implicated into interaction with large form of the growth factor [9]. Above-mentioned data suggest that each PDGF-BB variant may recognize and bind distinct GAG sequences. This possibility also results from other reports. Lustig et al. [28] found that short PDGF-BB displays significantly smaller affinity for low-molecular weight heparin than the long variant. Moreover, DS and CS were capable to compete with heparin or HS solely in the case of binding to short form of the growth factor [8,15]. The observed distinct GAG binding properties found for both PDGF-BB variants may result from the fact that they demonstrate different

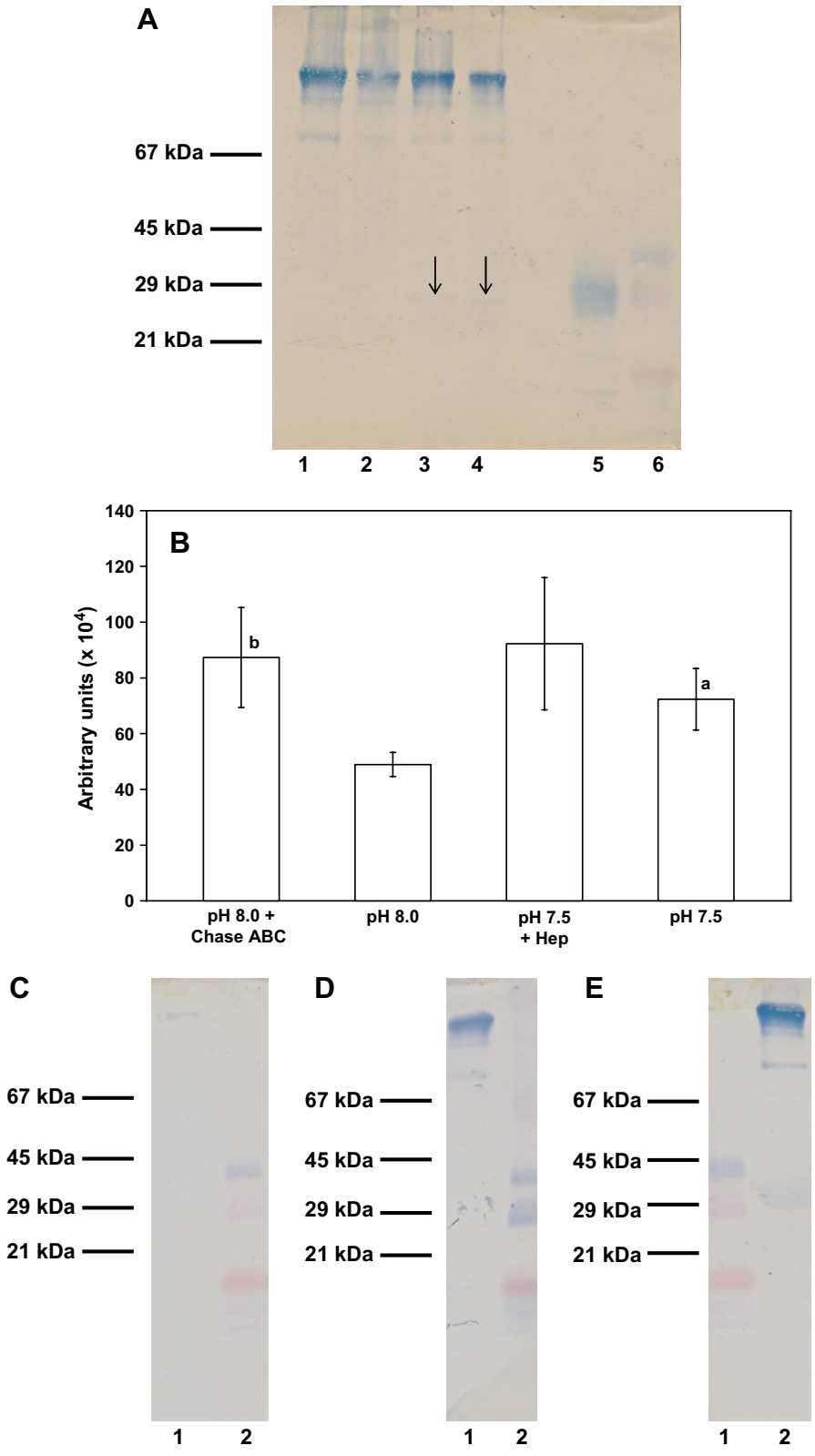

Fig. 8. Characteristics of PDGF-BB acceptors in DF. Samples of DF were extracted in the presence of chondroitinase ABC (Chase ABC) or heparinases I and III (Hep). Extract components were subsequently resolved by SDS-PAGE, and after electrotransfer to nitrocellulose membrane probed with various antibodies. The migration positions of the molecular weight markers are as indicated. (A) Blot probed with rabbit-anti-human PDGF-BB antibodies. Lane 1, DF extract, pH 8.0 with Chase ABC; lane 2, DF extract, pH 8.0 without Chase ABC; lane 3, DF extract, pH 7.5 with Hep; lane 4, DF extract, pH 7.5 without Hep; lane 5, recombinant human PDGF-BB; lane 6, electrotransfer markers. Arrows show position of short variant of PDGF-BB. (B) Total content of anti-human PDGF-BB serum reactive components released from DF by Chase ABC or Hep. Blots with immunoreactive components were subjected to densitometric analysis. Data represent the mean ± S.D. of extraction of five independent DF samples. a – differences statistically significant ($p < 0.05$) in relation to component content in DF extracts, pH 8.0 without Chase ABC; b – differences statistically significant ($p < 0.05$) in relation to component content in DF extracts, pH 8.0 without Chase ABC. (C) Blot probed with mouse monoclonal anti-human collagen type I IgG. Lane 1, DF extract, pH 8.0 with Chase ABC; lane 2, electrotransfer markers. (D) Blot probed with mouse monoclonal anti-human fibronectin IgG. Lane 1, DF extract, pH 8.0 with Chase ABC; lane 2, electrotransfer markers. (E) Blot probed with polyclonal rabbit-anti-human collagen type III antibodies. Lane 1, electrotransfer markers; lane 2, DF extract, pH 8.0 with Chase ABC.

configurations of polypeptide chain motifs potentially involved in the interaction. The long PDGF-BB monomers have two such motifs representing cationic amino acid residue clusters localized in both C-terminal region and the loop III while only the latter module is present in the short growth factor monomers [8,29].

Our present data have also revealed that in DF appreciable amount of PDGF-BB is bound to tissue GAGs especially DS/CS. It is not known which DF CS/DSPGs are responsible for the interaction in vivo. Our solid phase assay results suggest that both DF decorin and DF biglycan DSs are able to bind the growth factor with similar affinity. However, lower binding capacity of the latter GAG as compared to the former one indicates that only part of the tissue pool of biglycan can participate in the interaction with PDGF-BB.

Hugeness of PDGF-BB molecules released from DF by GAG depolymerizing enzymes represents components of supramolecular complexes composed of PG and other ECM molecules such as type III collagen or fibronectin covalently coupled to the growth factor. It seems that the cross-linking of PDGF-BB to above-mentioned fibrous glycoproteins can be very effective since only negligible amount of the growth factor was found to interact solely with GAGs, especially HS. The best candidates responsible for the formation of these complexes seem to be transglutaminases known to cross-link miscellaneous proteins through a reaction between the γ-carboxamide group of glutamine residue and the ε-amino group of lysine residue [30]. Moreover, both type III collagen and fibronectin are substrates for the enzymes [30] while the growth factor as containing some lysine residues can potentially participate in the cross-link reaction. Interestingly, increased activity of transglutaminases is found in DF [31]. What is a role of DSPG(s) in the formation of such molecular complexes involving PDGF-BB? From our study results that DF derived DSs displayed high affinity for the growth factor in the solid phase assay. Moreover, these interactions are stronger than estimated by the same method the growth factor binding to collagen [32]. Thus it is conceivable that small DSPG side chains serve as preliminary acceptors for PDGF-BB which is next presented to the contiguous ECM molecules and covalently linked in the enzymatic reaction. This cascade of events leading to immobilization of PDGF-BB in ECM may contribute to regulation of the growth factor bio-availability as well as to restriction of fibrosis process. On the other hand, Nili et al. [33] have found that bovine articular cartilage decorin is able to bind PDGF-BB but does not prevent the growth factor interactions with cell receptors. Nevertheless, decorin inhibits the growth factor stimulated β receptor phosphorylation as well as cell proliferation, migration and collagen synthesis all implicated in wound repair process [33]. Thus, observed by us in the present study strong binding of NPF decorin DS to the growth factor may be significant for proper course of fascia wound healing. This suggestion is supported by the finding that NFL characterized by the presence of decorin with relatively low affinity for the growth factor displays excessive scarring when injured [19].

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