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Proteoglycans in human burn hypertrophic scar from a patient with Ehlers–Danlos syndrome*

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ABSTRACT

Proteoglycans (PGs) from human burn hypertrophic scar of a patient with Ehlers–Danlos syndrome were extracted with 4M guanidinium chloride and purified by DEAE-cellulose chromatography. Differential ethanol precipitation of the PG fraction obtained after ion-exchange chromatography yielded two low mol.-wt. PGs, one rich in glucuronic acid (PG_{GLCA}; M_r 66 kDa) and the other rich in iduronic acid (PG_{IDOA}; M_r 48 kDa). In PG_{GLCA}, 84% of the glycosaminoglycan chains are composed of GlcA→GalNAc(SO_4) units, whereas in PG_{IDOA}, the chains contain 95% IdoA→GalNAc(SO_4) disaccharide units. Upon treatment with testicular hyaluronidase, the PGs gave different-sized oligosaccharides. Chondroitinase ABC digestion of PG_{GLCA} or PG_{IDOA} gave a single protein core (M_r ~20 kDa). The presence of glucosamine and sialic acid in PG_{GLCA} and PG_{IDOA} suggests that both contain *N*-linked oligosaccharides.

INTRODUCTION

Hypertrophic scar, abnormally healed human skin that extends beyond the confines of the original wound[1], is characterized by excessive deposition of dermal collagen[2], which forms bundles or tracts in a prominent, whorl-like arrangement, or in packed nodules, or both[3].

Ehlers–Danlos syndrome is one of the inherited connective tissue disorders associated with abnormal collagen synthesis. It is characterized by hyperelastic, easily bruised, and velvety-soft skin[4]. In addition to collagen abnormalities, changes occur in the structure of fibronectin[5] and low mol.-wt. proteoglycans (PGs)[6]. Small PGs consist of one or two glycosaminoglycan (GAG) chain(s) covalently linked to a protein core[7]. These macromolecules bind to specific amino acid residues in soluble collagen[8], and the growth of collagen fibrils *in vitro* has been demonstrated to be controlled by the collagen–PG interaction[9–11].

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The collagen bundles present in hypertrophic and keloid scar tissues contain excessive amounts of PGs. After injury, a minor alteration in the structure or distribution of PGs could influence the type of scar formation. For these reasons, we investigated the changes in the amount and structure of the PGs present in normal human skin[12] and in different types of post-burn scars[13–15]. In order to understand further the role of low mol.-wt. PGs in the fibrillogenesis of hypertrophic scars *in vitro*, the PGs present in the hypertrophic scar of a burn patient with Ehlers–Danlos syndrome have been isolated and characterized.

EXPERIMENTAL

Materials. — The source of the materials was as follows: Sepharose CL-4B from Pharmacia (Piscataway, NJ); guanidinium chloride from Schwartz/Mann (Cleveland, OH); Tris, and testicular and streptomyces hyaluronidase from Sigma (St. Louis, MO); urea from Malinckrodt (Paris, KY); diethylaminoethyl cellulose (DE-52) from Whatman (Kent, England); chondroitinase ABC from ICN (Liesle, IL), unsaturated 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose (ΔDI-OS); 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose 4-sulfate (ΔDI-4S); 2-acetamido-2-deoxy-3-*O*-4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose 6-sulfate (ΔDI-6S), and chondroitinase AC from Seikagaku (Kogyo, Japan) through Miles (Elkhart, IN); and cellulose acetate plates from Helena (Beaumont, TX). All other electrophoresis chemicals were purchased from Bio-Rad (Richmond, CA).

Burn hypertrophic scar tissue was obtained from a 7-year-old male patient with Ehlers–Danlos syndrome[4] by surgery at Shriners Burns Institute, Boston Unit. The age of the scar tissue following burn injury was 18 months, and the type of scar was characterized by histological examination[16].

Preparation of PGs. — The tissue (51.1 g) was defatted with acetone and dried (19.2 g). After removal of the epidermis by scratching with a blade, the tissue was ground in a Wiley Mill. The procedure and proteinase inhibitors used to extract were the same as described earlier[14,17]. PGs were adsorbed on a DEAE-cellulose (DE-52) column that was equilibrated with 6.0M urea in 50mM sodium acetate, pH 5.8 (buffer *A*). The column was washed with the same buffer and hyaluronan, and the PGs were eluted with linear gradients 0–0.2M NaCl and 0.2–2M NaCl in buffer *A*, respectively.

Fractionation of PGs to give PG_{IDOA} and PG_{GLCA}. — The PG fraction, obtained as described above, was dissolved in 4M guanidinium chloride–0.05M sodium acetate, pH 7.6, containing proteinase inhibitors and precipitated with various ethanol concentrations as described previously[13,14]. The materials precipitated at 30 and 40% ethanol concentrations contained only PG_{IDOA}. The fraction precipitated at 75% ethanol concentration contained PG_{GLCA} contaminated with hyaluronan.

Purification of PG_{GLCA} by DE-52 chromatography. — The 75% ethanol-precipitated PG fraction was refractionated on a cellulose DE-52 column (15 × 1 cm) with a linear gradient of 0.2–1M NaCl in buffer *A*. This fractionation gave pure PG_{GLCA}.

Sepharose CL-4B column chromatography of PG_{GLCA} and PG_{IDOA}. — PG_{GLCA} and PG_{IDOA} were fractionated on Sepharose CL-4B columns (143 × 1 cm), eluted with 0.5M sodium acetate, pH 5.8, buffer. The uronic acid-positive peaks were pooled, dialyzed against water, and lyophilized.

Electrophoresis. — Cellulose acetate plate electrophoresis. The PG_{GLCA} and PG_{IDOA} were analyzed by cellulose acetate plate (6 × 7.5 cm) electrophoresis as described earlier by Cappelleti *et al.*[19]. The plates were stained with Alcian Blue to detect PGs.

Poly(acrylamide) gel electrophoresis (PAGE). PG_{GLCA}, PG_{IDOA}, GAG chains, and oligomers released after testicular hyaluronidase treatment were examined by PAGE, followed by Alcian Blue staining as described earlier by Cowman *et al.*[20].

Sodium dodecyl sulfate poly(acrylamide) gel electrophoresis (SDS–PAGE). Samples of the core protein obtained after digestion of the PG with chondroitinase ABC were subjected to SDS–PAGE by the procedure of Laemmli[21] using 1% SDS–7% poly(acrylamide) gel electrophoresis and stained with Coomassie Blue.

Analysis of glycosaminoglycan (GAG) chains from PG_{GLCA} and PG_{IDOA}. — Isolation of GAGs. PG samples (1 mg) were treated with M $NaBH_4$–50mM NaOH (500 μL) under the conditions of Carlson[22]. The reaction mixture was neutralized with acetic acid, dialyzed, and lyophilized.

Determination of Δ-disaccharides. GAG chains, obtained from PG_{GLCA} and PG_{IDOA} (50 μg), were digested with chondroitinase ABC (0.3 unit) or chondroitinase AC (0.3 unit) in 0.5M Tris·HCl buffer (50 μL), pH 8.0, for 2 h at 37° (ref. 23). After incubation, aliquots (2–3 μL) of the digests were analyzed by cellulose acetate plate electrophoresis as described by Miyamoto *et al.*[24].

Analysis of GAG chain oligomers released after testicular hyaluronidase treatment. PG samples (1 mg) in 0.15M NaCl–0.01M sodium acetate, pH 5.0, buffer (1 mL) were treated with testicular hyaluronidase (50 units) for 1 h at 37° to yield GAG oligomers[25]. The digests were analyzed by PAGE for different-sized oligomers[20].

Characterization of PG_{GLCA}- and PG_{IDOA}-core proteins. — Each PG was digested separately with chondroitinase ABC in the presence of proteinase inhibitors as described by Oike *et al.*[26]. The digests were analyzed by SDS–PAGE for M_r values of core proteins[21].

Analytical procedures. — The protein content of the DE-52 column fractions was determined by measuring A_{280} and by the Lowry *et al.* reaction[27], the uronic acid content by the borate–carbazole reaction[28], the hexose content by the anthrone reaction[29] using D-galactose as the standard, and sialic acid content by the Jourdian *et al.* method[30]. The content of hexosamine was determined with an amino acid analyzer after hydrolysis of the sample in 4M HCl at 100° for 8 h. The sulfate content of PG_{GLCA} and PG_{IDOA} was determined by the Antonopoulos method[31]. Amino acid analysis was performed after hydrolysis with 6M HCl at 110° for 20 h, followed by derivatization with phenyl isothiocyanate[32]. The column used was an Altex 3-μm ODS, and elution was as described in Waters "Picatag" manual. The N-terminal amino acid sequence of the PG sample was determined with an Applied Biosystems model 477A gas-phase sequencer with on-line h.p.l.c. detection of Phth-amino acid[33].

RESULTS

Human burn hypertrophic scar tissue PGs from a patient with Ehlers–Danlos syndrome were prepared by extraction with 4M guanidinium chloride containing proteinase inhibitors. The extracted material was fractionated on a DE-52 column, as described previously[34], and the different fractions were pooled, dialyzed, and lyophilized (see Fig. 1 and Table I). Fractions A and B did not contain PGs, whereas Fraction C contained hyaluronan. Fraction D contained PGs and, in order to separate the different types of PGs present, this fraction was dissolved in 4M guanidinium chloride containing proteinase inhibitors, followed by precipitation with various amounts of ethanol (Table II). The fractions precipitated with 20 and 50% ethanol concentrations contained

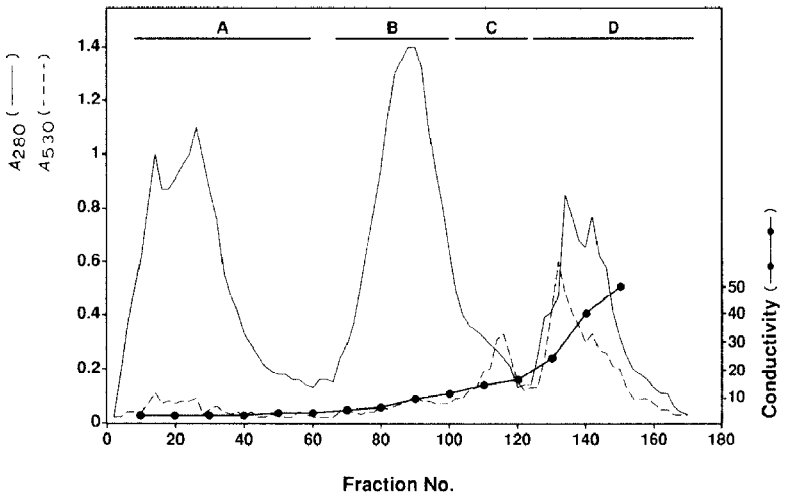

Fig. 1. DE-52 ion exchange chromatography of the 4M guaninidium chloride extract from the human Ehlers–Danlos syndrome hypertrophic scar. The column fractions (10 mL) were analyzed to determine A_{280}, the uronic acid content A_{530}, and the conductivity. Horizontal bars show the fractions pooled.

TABLE I

Distribution of different PGs in DE-52 column fractions

Fraction	*Yield (%)*[a]	*PG component detected*[b,c]				
		HP	*DS*	*HS*	*HA*	*CS*
A	64.7	–	–	–	–	–
B	23.3	–	–	–	–	–
C	5.5	–	–	–	+	–
D	6.5	(tr)	+	(tr)	(+)	+

[a] Yield (based on dry weight) of the material. [b] Abbreviations: HP, heparin; DS, dermatan sulfate; HS, heparan sulfate; HA, hyaluronan; and CS, chondroitin sulfate. [c] The presence or absence of an Alcian Blue-staining band with similar mobility to standard GAG is indicated with + or –; (+) indicates the presence of minor and (tr) a trace amount of GAG.

TABLE II

Yield and composition of various fractions obtained by ethanol precipitation

Ethanol conc. (%, v/v)	*Yield of PGs*		*PC component detected*[a]				
	Weight (mg)	*Percent of total recovered*	*HP*	*DS*	*HS*	*HA*	*CS*
20	2.54	3.6	+			tr	tr
30	17.44	24.7		+			
40	9.48	13.4		+			
50	9.20	13.0	+	(+)	(+)	(+)	(+)
75	32.00	45.3				(+)	+

[a] See footnotes (b) and (c) to Table I.

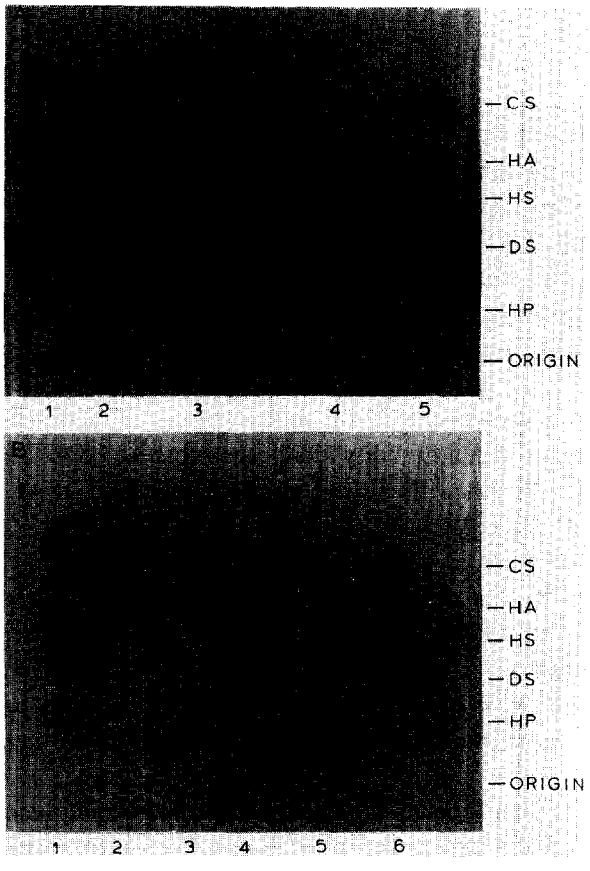

Fig. 2. Cellulose acetate electrophoresis of PG_{IDOA} (A) and PG_{GLCA} (B). In A and B, a mixture of reference GAGs (heparin [HP], dermatan sulfate [DS], heparan sulfate [HS], hyaluronan [HA], and chondroitin 4/6 sulfate [CS]), were applied to lanes 1 and 5, and 1 and 6, respectively. In A, PG_{IDOA}, digested with chondroitinase ABC and AC, was applied to lanes 2, 3, and 4, respectively. In B, PG_{GLCA}, digested with chondroitinase ABC and AC, was applied to lanes 2 and 5, 3, and 4, respectively.

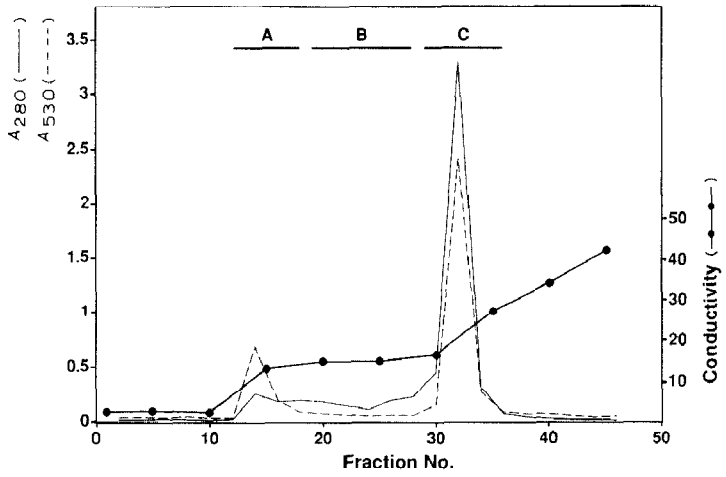

Fig. 3. DE-52 ion-exchange chromatography of the 75% ethanol-precipitated PG fraction. The column fractions were analyzed to determine A_{280}, the uronic acid content (A_{530}), and the conductivity. Horizontal bars show the fractions pooled.

heparin and other PGs, whereas those precipitated with 30 and 40% concentrations contained a single, Alcian Blue-staining band, which represents the iduronic acid-rich PG (PG_{IDOA}) with a mobility similar to standard DS·GAG. Examination of the PG_{IDOA} treated with chondroitinase ABC and AC (Fig. 2A, lanes 2–4) on cellulose acetate plate electrophoresis showed that PG_{IDOA} could only be degraded completely with chondroitinase ABC. The 75%-ethanol fraction contained glucuronic acid-rich PG_{GLCA} (with a mobility similar to standard CS·GAG) contaminated with hyaluronan. Pure PG_{GLCA} was obtained by fractionating the 75% ethanol-precipitated fraction on a DE-52 column with a linear gradient of 0.2–1 M sodium chloride in buffer *A* (Fig. 3). Fraction C had a single, Alcian Blue-positive band with a mobility identical to that of CS·GAG (Fig. 2B, lanes 2 and 5). Separate treatments with either chondroitinase ABC or AC completely eliminated this band (Fig. 2B, lanes 3 and 4, respectively).

Both PG_{GLCA} and PG_{IDOA} were purified on Sepharose CL-4B columns under associative conditions (Figs. 4A and B). The elution profiles of PGs contained single peaks of PG_{GLCA} and PG_{IDOA}, which were pooled, dialyzed, and lyophilized. The elution positions of PG_{GLCA} and PG_{IDOA} indicated M_r of 66 and 48 kDa, respectively. The M_r values of small PGs containing one or two GAG chains, determined earlier relative to proteins, were found to be similar to those determined by the sedimentation equilibrium method[35].

Both PG_{GLCA} and PG_{IDOA} were rich in leucine, as well as in aspartic and glutamic acid, serine, and glycine, and significant differences were noted for proline, and aspartic and glutamic acid (Table III). The overall pattern of amino acid composition is similar to that of hypertrophic scar[14] PG_{IDOA}.

In PG_{GLCA} ~83% of the GAG chains were composed of GlcA→GalNAc(SO_4) disaccharide repeating units, whereas in PG_{IDOA} only 5% of the GAG chains were

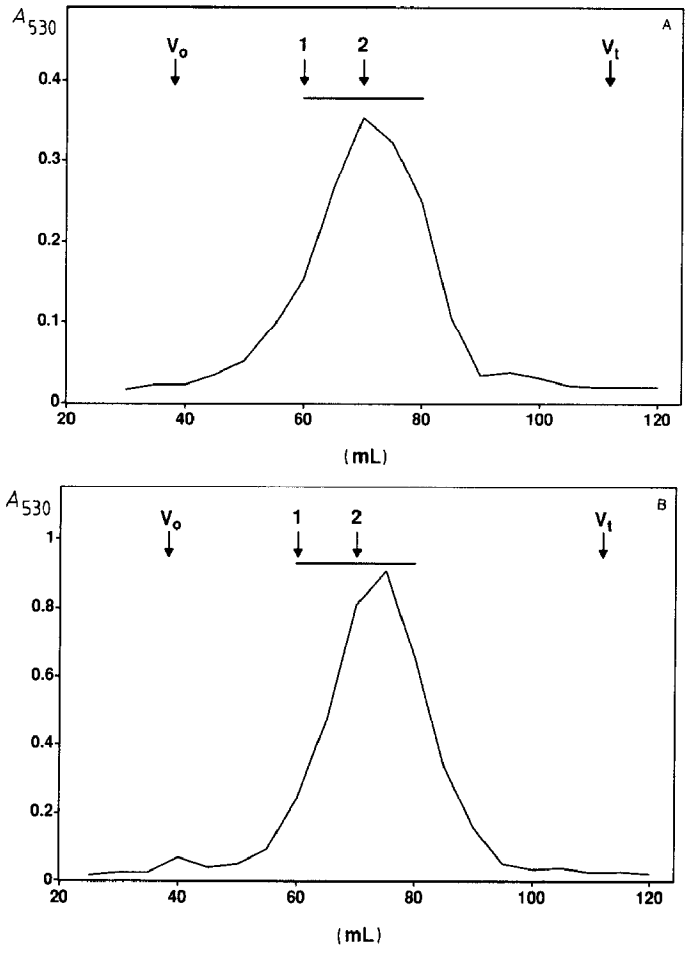

Fig. 4. Sepharose CL-4B gel chromatography of PG_{GLCA} (A) and PG_{IDOA} (B). Aliquots of the column fractions were analyzed for uronic acid content (A_{530}). The PG_{GLCA} and PG_{IDOA} were obtained by pooling the fractions shown by horizontal bars. The arrows marked 1, 2, V_o, and V_t indicate the elution positions of β-D-galactosidase (M_r 120 kDa), bovine serum albumin (M_r 66 kDa), Dextran Blue 2000, and uronic acid, respectively.

composed of GlcA→GalNAc(SO_4), and 95% were IdoA→GalNAc(SO_4) (Table IV). The presence of glucosamine (Table III) and sialic acid (Table IV) in PG_{GLCA} and PG_{IDOA} suggested that both PGs contained *N*-linked oligosaccharides. These oligosaccharides have previously been found in calf skin and human scar tissue[36,37] PGs. PG_{IDOA} is more sulfated than PG_{GLCA} (Table IV).

The M_r values of the PG_{GLCA} and PG_{IDOA} GAG chains, released by alkaline–borohydride treatment, were determined by poly(acrylamide) gel electrophoresis, followed by use of a logarithmic plot of the M_r values of standard GAGs from human normal and hypertrophic scar[14] (Fig. 5). The molecular mass of PG_{GLCA} and PG_{IDOA} GAG chains is 27 and 24.8 kDa, respectively.

TABLE III

Amino acid composition and hexosamine ratios of PG_{GLCA} and PG_{IDOA}

Amino acids	*Residues/1000 residues*	
	PG_{GLCA}	PG_{IDOA}
Aspartic acid	63	96
Threonine	90	50
Serine	160	137
Glutamic acid	112	118
Proline	66	90
Glycine	120	130
Alanine	83	73
Half-cystine	[a]	[a]
Valine	56	60
Methionine	9	8
Isoleucine	31	24
Leucine	58	73
Tyrosine	19	16
Phenylalanine	32	32
Lysine	33	31
Histidine	16	16
Arginine	22	31
Ratios of hexosamines		
Glucosamine	1	1
Galactosamine	6.7	19.8

[a] Not detected.

TABLE IV

Chemical composition of PG_{GLCA} and PG_{IDOA}

Components	PG_{GLCA}	PG_{IDOA}
Repeating dissaccharide units		
IdoA→GalNAc(SO_4)[a]	17	95
GlcA→GalNAc(SO_4)[a]	83	5
Hexuronic acid[b]	17.0	23.6
Hexoses[b]	1.86	1.56
Sialic acid[b]	1.29	0.42
Sulfate[b]	9.0	20.5
Protein[b]	10.0	10.0
Δ-Disaccharide[c]		
Δ-DI-4S	85	98
Δ-DI-6S	14	<1
Δ-DI-OS	<1	<1

[a] Percentage of IdoA→GalNAc(SO_4) or GlcA→GalNAc(SO_4), relative to total GAG, determined from the amounts of unsaturated disaccharides generated by digestion with chondroitinase ABC and AC. [b] Percentage of the dry weight of PG by procedures described in the Experimental section. [c] Percentage of the total Δ-disaccharides.

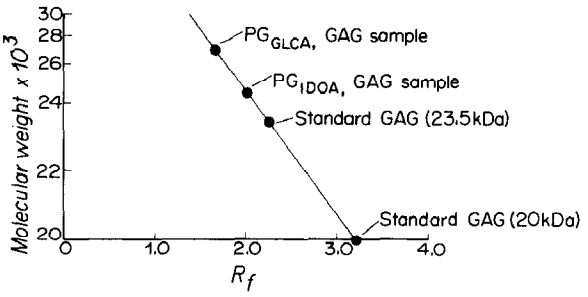

Fig. 5. Molecular-weight determination by PAGE of GAG chains obtained after treatment of PG_{IDOA} and PG_{GLCA} with alkaline borohydride.

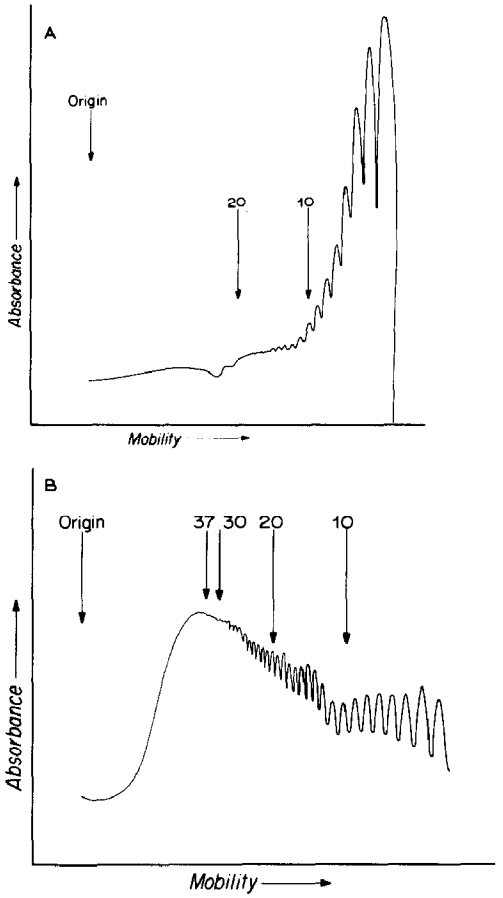

Fig. 6. Densitometric PAGE electrophoresis of PG_{GLCA} (A) and PG_{IDOA} (B) after testicular hyaluronidase treatment. The arrows marked 10, 20, and 30 indicate the mobilities of oligosaccharides that contain 10, 20, and 30 disaccharide units.

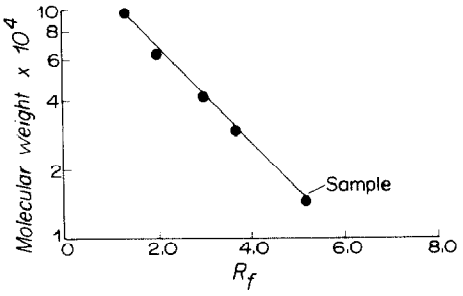

Fig. 7. Molecular-weight determination of PG_{IDOA} protein core obtained after PG_{IDOA} digestion with chondroitinase ABC.

The distribution of L-iduronic acid in PG_{GLCA} and PG_{IDOA} was examined by testicular hyaluronidase treatment (Fig. 6). The highest mol.-wt. oligosaccharide from PG_{IDOA} contained >37 disaccharide units and that from PG_{GLCA} 20 disaccharide units. These results are in agreement with the results of chondroitinase ABC and AC treatments and confirmed that PG_{IDOA} is rich in L-iduronic acid and PG_{GLCA} in D-glucuronic acid.

The GAG chains of PG_{IDOA} were removed by chondroitinase ABC digestion, and the M_r value of the protein core was determined by SDS–PAGE by plotting the mobility of the protein core against the logarithmic M_r values of standard proteins, namely, phosphorylase (92 500), bovine serum albumin (66 200), ovalbumin (45 000), and carbonic anhydrase (31 000) (Fig. 7). The core protein was small and had the M_r value of ~14 kDa; the M_r value of the PG_{GLCA} protein core was similar (data not shown). The protein core of normal human hypertrophic scar was reported[14,37] to contain two peptide chains with M_r values ranging from 17 to 30 kDa. In one study[37] where the whole skin was used without scraping the epidermis, an additional protein core band having M_r 53 kDa was also observed. Two possible reasons for the small protein core of EDS-PGs could be the degradation of PGs during the extraction of the tissue or a defect in the synthesis of the protein core. The first possibility was investigated earlier[14] by use of several extraction procedures; all of them gave PGs of the same size[12]. Moreover, a recent study[12] clearly demonstrated that the human epidermis PG protein core has a high M_r (~45 kDa), as compared to the dermis PG protein core M_r (~ 20 kDa). A second, more likely possibility is that the synthetic mechanism for the core protein in the hypertrophic scar is defective, as is the case for normal skin in Ehler–Danlos syndrome patients.

The *N*-terminal amino acid sequence of PG_{IDOA} is similar to that reported for human skin or scar[12,14], and bone PG-II[38]. Viscosity experiments with standard hyaluronan (data not given) suggested that neither PG_{GLCA} nor PG_{IDOA} form aggregates.

DISCUSSION

The structural characteristics of small PGs from different connective tissues were summarized recently (see Table VII, ref. 7). The physiological roles of small PGs are unknown, but these macromolecules bind to collagen fibrils[39,40] or to fibronectin[41] (or both), and inhibit collagen fibrillogenesis[9]. Several studies[13–15] from our laboratory have demonstrated that, in human burn scarring, changes in the structure and distribution of PGs occur. On this basis, we hypothesized that alterations in collagen organization in burn scar tissues may result from modification of small PG structures.

Ehlers–Danlos syndrome is a disease involving defective packing of collagen fibrils into fibers, resulting in hyperextensibility and fragility. The functional abnormality of the cutaneous tissue was reported to be due to a molecular defect in PGs[6]. Excessive deposition of dermal collagen bundles and their associated PGs in burn hypertrophic scars were observed earlier[3]. Therefore, to understand further the role of skin small PGs in maintaining the skin's structural integrity, particularly in scarring, the structure of small PGs from human burn hypertrophic scar with Ehler–Danlos syndrome were examined. Two types of small PGs, PG_{IDOA} and PG_{GLCA}, were present in this hypertrophic scar tissue. As a result of this study, it can be concluded that PGs from burn hypertrophic scar of the Ehler–Danlos syndrome patient differ from those of similar scars from normal individuals (Table V). We found differences in the size of the GAG chains, their degree and position of sulfation, and the ratio of types of PGs containing D-glucuronic *vs.* L-iduronic acid[13,14]. These results also suggested a defect in the synthesis of the Ehler–Danlos syndrome PG protein core, as the size of the PG protein cores was found to be smaller in comparison to the PG protein core of hypertrophic scar from normal burn patients[14]. In conclusion, the results presented herein support earlier findings[6,14] that human PGs with apparent abnormalities in their

TABLE V

Characteristics of small PGs from burn hypertrophic scar of Ehlers–Danlos syndrome patient and normal individuals

	PG_{IDOA}		PG_{GLCA}	
	Normal[a]	*EDS*	*Normal*[b]	*EDS*
Mol.-wt. (kDa)				
PG		48	78	66
GAG chain	23.5	24.8	29.0	27.0
Core protein	21.5,17.0	20.0	22,16	20.0
IdoA (%)	89.5	95.0	13.0	17.0
Uronic acid				
4-SO_4: 6-SO_4 groups	15.5	98	1.4	6.1
Number of disaccharide units present in the highest mol-.wt. oligosaccharide obtained after treatment with testicular hyaluronidase	47	36	25	20

[a] Ref. 14. [b] Ref. 13.

structure play a role in the formation of an aberrant skin architecture and its morphological consequences.

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